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PHOSPHOLIPID REMODELING IN THE RAT HEPATOCYTE

by

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled PHOSPHOLIPID REMODELING IN THE RAT HEPATOCYTE submitted by Rockford William Samborski in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

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DEDICATION

We are born alone and we die alone, but as we travel the distance between these two events we are seldom, if ever, alone. Thus, this thesis is dedicated to those who have influenced and supported me during these studies. Above all, I would like to dedicate this thesis to the memory of Mr. Ken Williams, for it is he who taught me the reason for medical research. This thesis is also dedicated to my family; Andy, Lynn, Nadine, Roseanne, Danny, and Joe, for it is they who feed me when the Heart and Stroke Foundation money ran low. This thesis is also dedicated to Dalene, thanks for making Edmonton winters seem a bit warmer. Finally, I would like to dedicate this thesis to all the scientists, who labored in building the foundations upon which this thesis rests.

ABSTRACT

The metabolism of molecular species of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) have been studied in cultured rat hepatocytes. Newly-made PC molecular species, derived from either [^{3}H]choline or [^{3}H]ethanolamine, undergo a series of remodeling reactions with 18:0-20:4-PC being the final product. Remodeling of the PC species involves both the *sn*-1 and *sn*-2 acyl groups of the phospholipid molecule. These remodeling reactions occur in the absence of PE methylation and appear to be regulated by the level of cellular choline. In contrast, the newly-made PC molecular species, derived from [^{3}H]serine, do not appear to be remodeled. These results support the view that metabolically distinct pools of PC exist in the hepatocyte.

The molecular species of PE, synthesized from [^{3}H]ethanolamine on the endoplasmic reticulum, are not significantly remodeled in the hepatocyte. The major metabolic fate of [3H]ethanolamine-labeled PE is the conversion to PC, by methylation. If not converted to PC, the PE is rapidly catabolized by phospholipases. 16:0-22:6-PE has a turnover rate which is 2-3 fold greater than that for any other major PE species. Inhibition of PE methylation, with the drug 3-deazaadenosine (DZA), increases the amount 16:0-22:6-PE which is degraded by phospholipase(s) but has little affect on any other major PE species. These results suggest that a phospholipase competes with the methylation pathway for the newly-made 16:0-22:6-PE. In contrast, the molecular species of PE, derived from [^{3}H]serine, appear to be remodeled in the hepatocyte. These findings suggest that the site of phospholipid biosynthesis plays a major role in determining the fate of newly-synthesized phospholipids.

We have found that the CDP-choline pathway has the potential to provide the hepatocyte with all the major PC species. Prolonged inhibition of the methylation pathway with DZA does not alter the composition of cellular PE or PC. Furthermore, newly-made 16:0-22:6-PC, derived from [^{3}H]ethanolamine, is rapidly remodeled in the hepatocyte. These findings suggest that PE methylation is not responsible for the generation of a specific 'hexaenoic-rich' pool of PC in the hepatocyte.

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TABLE OF CONTENTS

CHAPTER PAGE

INTRODUCTION.......1
- I. GENERAL OVERVIEW OF BIOLOGICAL MEMBRANES.......1
  - I.A. Phospholipid Diversity and Distribution.......1
  - I.B. Membrane Lipid Composition.......3
  - I.C. Structure of Glycerophospholipids.......5
  - I.D. Function of Phospholipids.......7
- II. BIOSYNTHESIS OF PHOSPHOLIPIDS.......10
  - II.A. The *de novo* Biosynthesis of PA, DG, and CDP-DG.......10
  - II.B. PC Biosynthesis.......13
  - II.C. PE Biosynthesis.......16
  - II.D. PS Biosynthesis.......18
  - II.E. 1-alkyl- and 1-alkenyl-Phospholipid Biosynthesis.......18
- III. REMODELING OF PHOSPHOLIPIDS.......19
  - III. A. Deacylation of Phospholipids.......21
    - III. A.I. Phospholipase A_1.......21
    - III. A.II. Phospholipase A_2.......25
    - III. A.III. Lysophospholipase.......32
  - III. B. Reacylation of Phospholipids.......32
    - III. B.I. Acyl CoA synthetases.......32
    - III. B.II. Acyl-CoA: lysophospholipid acyltransferase.......33
    - III. B.III. CoA-Independent Transacylases.......35
    - III. B.IV. LysoPC: LysoPC transacylase.......36
- IV. RATIONALE AND OBJECTIVES OF THESIS.......36
- BIBLIOGRAPHY.......52

CHAPTER II. 73

METABOLISM OF [^{3}H]ETHANOLAMINE-LABELED PE AND PC 73

INTRODUCTION 73

MATERIALS AND METHODS 74

Materials 74

Animals 74

Hepatocyte culturing 74

Pulse chase experiments 75

Lipid extraction and isolation of PE and PC 75

HPLC analysis of PC and PE molecular species 76

RESULTS 77

Metabolism of [^{3}H-methyl]Choline-Labeled PC 77

Metabolism of [^{3}H]ethanolamine-labeled PE 77

Remodeling of [^{3}H]Ethanolamine-Labeled PC but not [^{3}H]Ethanolamine-Labeled PE 79

DISCUSSION 81

Newly Synthesized PE is the Substrate for Methylation to PC 81

Remodeling of the Molecular Species of PC, but not PE 82

BIBLIOGRAPHY 114

CHAPTER III. 117

ROLE OF PEMT IN THE METABOLISM OF PE AND PC 117

INTRODUCTION 117

MATERIALS AND METHODS 119

RESULTS 120

DISCUSSION 124

BIBLIOGRAPHY 154

CHAPTER IV.. 158

SUBCELLULAR CATABOLISM OF [^{3}H]ETHANOLAMINE-LABELED PE IN RAT LIVER 158

INTRODUCTION 158

MATERIALS AND METHODS 159

RESULTS 161

DISCUSSION 164

BIBLIOGRAPHY 180

CHAPTER V 183

METABOLISM OF PC IN CHOLINE DEFICIENT RAT HEPATOCYTES 183

INTRODUCTION 183

MATERIALS AND METHODS 184

RESULTS 185

DISCUSSION 187

BIBLIOGRAPHY 204

CHAPTER VI. 207

METABOLISM OF [^{3}H]SERINE-LABELED PE AND PC IN RAT HEPATOCYTEs 207

INTRODUCTION 207

MATERIALS AND METHODS 209

RESULTS 211

DISCUSSION 217

BIBLIOGRAPHY 241

CHAPTER VII 245

DISCUSSION 245

BIBLIOGRAPHY 249

LIST OF TABLES

TABLE **PAGE**

II.1. Percent distribution of [^{3}H]choline-labeled PC molecular species in hepatocytes as a function of time 105

II.2. Catabolism of [^{3}H]ethanolamine-labeled PE during a 12-h chase 107

II.3. Percent distribution of [^{3}H]ethanolamine-labeled PE molecular species in hepatocytes as a function of time 109

II.4. Turnover times and turnover rates of PE molecular species 111

II.5. Percent distribution of [^{3}H]ethanolamine-labeled PC molecular species in hepatocytes as a function of time 113

III.1. Catabolism of [^{3}H]ethanolamine-labeled PE during a 12-h chase in the absence of 10 µM DZA 149

III.2. Catabolism of [^{3}H]ethanolamine-labeled PE during a 12-h chase in the presence of 10 µM DZA 151

III.3. Metabolism of [^{3}H]ethanolamine-labeled PE molecular species in control and DZA-treated cells 153

IV.1. Characterization of [^{3}H]ethanolamine-labeled microsomal and mitochondrial fractions 175

IV.2. Percent distribution of [^{3}H]ethanolamine-labeled PE molecular species in microsomes as a function of time 177

IV.3. Percent distribution of [^{3}H]ethanolamine-labeled PE molecular species in mitochondria as a function of time 179

V.1. Characterization of PC species from the liver of CD and CS rats 199

V.2. Metabolism of [*methyl*-^{3}H]choline-labeled PC species in CD hepatocytes 201

V.3. Metabolism of [*methyl*-^{3}H]choline-labeled PC species in CS hepatocytes 203

VI.1. Catabolism of [^{3}H]serine during a 4-h chase 238

VI.2. Distribution of [^{3}H]serine derived label in cellular PS, PE and PC 240

LIST OF FIGURES

FIGURE		PAGE
I.1.	Phospholipid composition of rat liver membranes	39
I.2.	The fluid mosaic model	41
I.3.	General structure of a glycerophospholipid	43
I.4.	de novo biosynthesis of PA, DG and CDP-DG	45
I.5.	Major pathways for PC biosynthesis	47
I.6.	Major pathways for PE biosynthesis	49
I.7.	Potential pathways for phospholipid remodeling	51
II.1.	Typical HPLC elution profile of rat liver microsomal PC	87
II.2.	Specific radioactivity of PC species derived from [^{3}H]choline	89
II.3.	Total radioactivity of PE and PC derived from [^{3}H]ethanolamine (HPLC preparation)	91
II.4.	Total radioactivity of PE and PC derived from [^{3}H]ethanolamine (analytical preparation)	93
II.5.	Specific radioactivity of PE species derived from [^{3}H]ethanolamine	95
II.6.	Decay of radioactivity from [^{3}H]ethanolamine-labeled PE and PC species	97
II.7.	Specific radioactivity of PC species derived from [^{3}H]ethanolamine	99
II.8.	Remodeling of [^{3}H]ethanolamine-labeled PC species (16:0-22:6; 18:0-20:4)	101
II.9.	Remodeling of [^{3}H]ethanolamine-labeled PC species (16:0-20:4; 16:0-18:2 & 18:1-18:2; 18:0-22:6; 18:0-18:2; 18:0-22:5)	103
III.1.	10 µM DZA slightly increases the mass of cellular PE, but not PC in hepatocytes	131
III.2.	10 µM DZA does not alter the major molecular species of PE	133
III.3.	10 µM DZA does not alter the major molecular species of PC	135

FIGURE		PAGE
III.4.	Effect of 10 µM DZA on the total radioactivity of cell PE and PC derived from [^{3}H]ethanolamine	137
III.5.	Effect of 10 µM DZA on the specific radioactivity of molecular species of PE derived from [^{3}H]ethanolamine (part 1)	139
III.6	Effect of 10 µM DZA on the specific radioactivity of molecular species of PE derived from [^{3}H]ethanolamine (part 2)	141
III.7.	Effect of 10 µM DZA on the specific radioactivity of molecular species of PC derived from [^{3}H]ethanolamine (part 1)	143
III.8	Effect of 10 µM DZA on the specific radioactivity of molecular species of PC derived from [^{3}H]ethanolamine (part 2)	145
III.9.	An hypothetical docosahexaenoate cycle involved in the biosynthesis of PC	147
IV.1.	Catabolism of microsomal [^{3}H]ethanolamine-labeled PE by endogenousphospholipase activity	167
IV.2.	Specific radioactivity of microsomal PE species derived from [^{3}H]ethanolamine	169
IV.3.	Catabolism of mitochondrial [^{3}H]ethanolamine-labeled PE by endogenousphospholipase activity	171
IV.4.	Specific radioactivity of mitochondrial PE species derived from [^{3}H]ethanolamine	173
V.1.	Specific radioactivity of molecular species of [^{3}H]choline-labeled PC isolated from CD hepatocytes	193
V.2.	Specific radioactivity of molecular species of [^{3}H]choline-labeled PC isolated from CS hepatocytes	195
V.3.	Hypothetical model for the transformation of 16:0-18:1-PC to 18:0-20:4-PC	197
VI.1.	Specific radioactivity of cellular PS, PE and PC derived from [^{3}H]serine	222
VI.2.	Distribution of [^{3}H]serine in cell and medium of hepatocytes	224
VI.3.	Specific radioactivity of cellular PS, PE and PC derived from [^{3}H]serine	226
VI.4.	Specific radioactivity of head group of cellular PS, PE and PC derived from [^{3}H]serine	228

FIGURE		PAGE
VI.5.	Percent distribution of radioactivity of PE molecular species derived from [^{3}H]serine	230
VI.6.	Specific radioactivity of head group of PE molecular species derived from [^{3}H]serine	232
VI.7.	Percent distribution of radioactivity of PC molecular species derived from [^{3}H]serine	234
VI.8.	Specific radioactivity of head group of PC molecular species derived from [^{3}H]serine	236

ABBREVIATIONS

ACLAT,	acyl-CoA:lysophospholipid acyltransferase
BSA,	bovine serum albumin
Ca^{2+},	divalent calcium
CD,	choline deficient rat
CS,	CD hepatocytes supplemented with choline and methionine
CDP-DG,	cytidine-5'-diphosphate-DG
CK,	Choline kinase
CoA,	coenzyme A
CPT,	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CT,	CTP: phosphocholine cytidylyltransferase
CTP,	cytidine-5'-triphosphate
DG,	diacylglycerol
DHAP,	dihydroxyacetone phosphate
DZA,	3-deazaadenosine
E,	ethanolamine
E-P,	ethanolaminephosphate
EK,	ethanolamine kinase
EPT,	CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase
ET,	CTP:phosphoethanolamine cytidylyltransferase
G-3-P,	*sn*-glycerol-3-phosphate
GPE,	glycerolphosphoethanolamine
GPS,	glycerolphosphoserine
HPLC,	high pressure liquid chromatography
IP_3,	inositol-3-phosphate
kD,	kilodalton
lysoPA,	either 1-acyl- or 2-acyl-*sn*-glycerophosphatidic acid
MEM,	Dulbecco's modified Eagles medium
PA,	phosphatidic acid
PACT,	PA: cytidylyltransferase
PAF,	platelet-activating factor

PAP,	phosphatidate phosphohydrolase
PC,	phosphatidylcholine
PE,	phosphatidylethanolamine
PEMT,	phosphatidylethanolamine-N-methyltransferase
PG,	phosphatidylglycerol
PS,	phosphatidylserine
PI,	phosphatidylinositol
PIP$_2$,	phosphatidylinositol 4,5-bisphosphate
PKC,	Ca^{2+}-activated, phospholipid dependent protein kinase
PLA$_1$,	phospholipase A$_1$
PLA$_2$,	phospholipase A$_2$
PLC,	phospholipase C
PLD,	phospholipase D
S,	serine
S-P,	serine phosphate
sn,	stereospecificity numbering
TG,	triacylglcerols
TLC,	thin layer chromatography

CHAPTER I.

INTRODUCTION

I. General Overview of Biological Membranes

Biological membranes are found in all but the simplest living organisms (ie. the non-membrane encapsulated viruses). One of the earliest recognized function of a cell membrane was to provide a physical barrier separating the cytosol from the external milieu. In prokaryotes, the membrane can act as a matrix to which proteins are associated and thus lead to metabolic compartmentation. In eukaryotes, the occurence of membrane bound organelles adds a further levels of compartmentation. However, cellular membranes are much more than just quiescent barriers. Due to its selective permeability, membranes have the capacity to store energy in the form of either ion or pH gradients. Membranes phospholipids can also act as sources of 'second messengers' which mediate the transfer of information from the external environment to the inside of the cell. Thus, it is clear that membranes are extremely dynamic.

The dynamic nature of the membrane is a reflection of the properties of the constituent lipids. There exist a great variety of lipids in membranes, of which phospholipids make up the largest group. Membrane phospholipids are constanly being synthesized, remodeled, and degraded. It is the purpose of this investigation to examine the dynamic nature of phospholipids in the membranes of primary cultures of rat liver hepatocytes. This thesis describes a series of experiments which utilize radiolabeled phospholipid precursors to study the fate of newly synthesized phospholipid molecular species. The following pages in this introductory chapter are intended to provide the reader with an adequate background to evaluate the experimental results and conclusion presented in this thesis.

I.A. Phospholipid Diversity and Distribution

I.A.I. Definition of Phospholipids

What is a phospholipid? The definition of a lipid most commonly used is "any biological material soluble in organic solvents" (Cullis and Hope, 1985). Thus, using this definition we can further define any lipid containing a phosphorus moiety as a phospholipid. There are two major classes of phospholipids, the glycerophospholipids and the sphingolipids. Sphingolipids utilize sphingosine as a back bone while the glycerophospholipids employ glycerol. While there are many other lipids in the cell besides phospholipids the central focus of this thesis is the metabolism of glycerophospholipids. Thus, the following discussion will be limited to phospholipids, with particular emphasis on the glycerophospholipids.

I.A.II. Distribution of Phospholipids in Nature

In most animals the glycerol-based phospholipids predominate: these include; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidic acid (PA) and diphosphatidylglycerol or cardiolipin (Cullis and Hope, 1985). Sphingosine and glycosphingolipids, the sphingosine-based lipids, are also present and may constitute a major or minor fraction of the total cell phospholipids. Fig. I.1. shows the phospholipid composition of membranes from rat liver (Daum, 1985; Rothman and Kennedy, 1977).

As indicated in Fig. I.1., the levels of individual phospholipids may vary considerably from membrane fraction to membrane fraction. Typically, cardiolipin is usually found only in the inner mitochondrial membrane while sphingomyelin is concentrated in the plasma and lysosomal membrane fractions. More will be said about the composition of mammalian membranes in section I.B.

In bacteria, the major phospholipids are PG, PE and cardiolipin. PC, PI and sphingolipids are not usually present. The levels of PG may vary considerably from species to species. For example, in cyanobacteria PG is the only phospholipid synthesized whereas it is completely absent in mycobacteria (Gunstone et al. 1986). PE is most commonly the major phospholipid of gram-negative bacteria and some gram-positive species (Gunstone et al. 1986). In contrast to the animals and plants, where cardiolipin is usually found only in the inner membrane of the mitochondria, cardiolipin is a major constituent of the bacterial membrane. Interestingly, sphingomyelin has been shown to account for between 6 and 62% of the total phospholipids in trypanosomes (Godfrey, D.G., 1967). For a comprehensive overview on the genetics and regulation of bacterial lipid metabolism the reader is directed to a recent review (van den Boom and Cronan, 1989).

In plants, the major phospholipids are PC, PE, PS, PI, PG and cardiolipin (Moore, 1982). The sphingolipids are also present, but in very low levels. The relative levels of phospholipid classes vary from one membrane source to another. For example, cardiolipin is almost exclusively found in the mitochondria (Bligny and Douce, 1980), whereas PG is highly enriched in the inner membranes of chloroplasts (Harwood, 1980). For further information on the metabolism of plant phospholipids the reader is directed to several reviews (Harwood, 1979; Moore, 1982).

I.B. Membrane Lipid Composition

I.B.I. Can We Predict Membrane Lipid Composition?

The composition of membranes within a single cell type have been shown to vary considerably (Fig. I.1.). In addition, the phospholipid composition of erythrocyte membranes from rat, human, and bovine sources also differ significantly (van Deenen and de Gier, 1974). In contrast, the phospholipid composition of liver membranes from rat, sheep, frog, mouse and bovine sources are remarkably similar (Rouser et al., 1968). Membrane composition may also be varied by dietary manipulations (Sjoblom, 1990). Clearly, the composition of any cellular membrane will be determined by a number of factors including: intracellular membrane location, specific membrane function, species type, environment (ie pH, temperature etc.), and diet. Thus, there appears to be no set rule for predicting what the phospholipid composition of a given membrane will be.

I.B.II. Are Membranes Homogeneous Bilayers?

It is now almost twenty years since Singer and Nicholson (1972) proposed the 'Fluid mosaic model' of a cell membrane (Fig. I.2.). The central feature of this model is the arrangement of the phospholipids into a bilayer consisting of an inner and outer leaflet. It is now well accepted that that the inner and outer leaflets of membrane bilayers have different lipid composition (op den Kamp, 1979; Bishop and Bell, 1988). With respect to the erythrocyte membrane, PE, PS and PI are found mainly in the inner leaflet, whereas PC and sphingomyelin are localized to the outer leaflet. Post et al. (1988) have recently described the phospholipid asymmetry that exist in rat cardiac sarcolemma. They report that essentially all of the PS and PI are on the inner leaflet while 75%, 7% and 57% of PE, sphingomyelin and PC, respectively, are found on the inner leaflet.

The mechanism by which membrane asymmetry is generated and maintained is not well established but appears to involve a number of complex processes. These processes probably involve the synthesis of new membrane lipids in the Golgi body and endoplasmic reticulum (reviewed in: Bishop and Bell, 1988). The processess which regulate the 'flip-flopping' of phospholipids across the membrane may also contribute to membrane asymmetry. There is growing evidence that the asymmetric distribution of PS in the red blood cell membrane is maintained by both ATP-dependent translocation (Tilley et al. 1986), and a direct interaction with membrane skeletal proteins (Middelkoop et al. 1988). Using rabbit renal brush-border membranes, Venien et al. (1988) have implicated aminophospholipid-cytoskeletal protein interactions in the maintenance of the lipid asymmetry in these membranes. They also suggest that sphingomyelin may play a role in this mechanism. There is also the recent suggestion that some membrane PC molecules may interact with the sialyl residue of sialocompounds and that this may contribute significantly to the maintenance of the phospholipid asymmetry in brain microsomes (Freysz et al., 1986). Finally, the action of both intracellular and extracellular phospholipases may also contribute to the overall maintenance of membrane asymmetry. For a more comprehensive treatment on the assembly and maintenance of asymmetric bilayers the reader is referred to a recent review (Bishop and Bell, 1988).

I.B.III. Phospholipid Molecular Species

Each class of phospholipid is composed of a number of molecular species, which differ in their fatty acid moieties. Analysis of rat liver phospholipids, by high pressure liquid chromatography (HPLC) has demonstrated the presence of greater than 100 different molecular species (Patton et al., 1982). The proportion of each molecular species differs quite dramatically among individual classes isolated from the same source. For example, PE isolated from human red blood cells contain 12.9 mole% palmitate and 23.7 mole% arachidonate, while PC, isolated from the same samples, contains 31.2 and 6.7 mole% of palmitate and arachidonate, respectively (Cullis and Hope, 1985). Furthermore, it is well documented that there exists a pattern of sexual dimorphism, with respect to molecular species, in the rat (reviewed in: Oscarsson and Eden, 1988). Characteristically, a higher level of arachidonic and stearic acids, and a lower level of palmitic acid has been found in PC and PE isolated from the liver, heart, plasma and erythrocytes of female rats compared to male rats.

Further membrane complexity is created by the occurrence of an 1-O-alkyl or an 1-O-alkenyl ether-linked molecular species. For example, while rat liver PE is essentially all 1-acyl-linked (Vance, J. E., 1990), the PE in human erythrocytes consists of equal portions of 1-acyl- and 1-alkenyl-linked molecules (Myher et al., 1989). Thus while the liver PE consists of over 32 molecular species the erythrocyte contains greater than 2-fold more PE species.

As with individual classes of phospholipids, there also appears to be asymmetric transbilayer distribution of molecular species. For example, the monoene species of PE are particularly enriched in the outer leaflet of the human erythrocyte, where they comprise approximately 30% of the PE species (Hullin, et al., 1991). In these same membranes, the polyunsaturated species were enriched in the inner leaflet. In trout brush-border membranes, 64% of PE and 69% of PS are located in the inner leaflet and are enriched in docosahexaenoic acid compared to the outer leaflet (Pelletier et al., 1987).

In summary, the cellular membranes are complex structures which consist of a non-random arrangement of lipids. Because of this complexity it has been impossible to attribute specific functions to individual lipid molecule. However, with the recent improvements in the methods for analyzing complex mixtures of lipids it may now be possible to elucidate specific functions for individual molecular species.

I.C. Structure of Glycerophospholipids

From a structural point of view phospholipid molecules are best viewed as being a molecule derived from three basic building blocks. These building blocks are: a backbone, consisting of a phosphoric ester of glycerol, fatty acid ester(s), and a hydrophilic head group (most commonly a nitrogenous base). Fig. I.3. is a cartoon showing the general structural features of a glycerophospholipid.

The glycerophospholipid molecule contains an asymmetric carbon which can be named either L-glycerol 3-phosphate or D-glycerol 1-phosphate To avoid problems with the nomenclature of glycerol derivatives the stereospecific numbering (*sn*) system was introduced. In this system the carbon atom of the glycerol molecule, furthest removed from the phosphate ester, is referred to as the *sn*-1 position.

While there are a vast number of different fatty acids available for incorporation into membrane phospholipids only a small percent are actually used. Most commonly these fatty acids have an even number of carbon atoms varying from 14 to 24 carbons in length and may be either saturated or unsaturated. Usually, the unsaturated fatty acids found in biological membranes may have from one to six double bonds, which are invariably in the cis configuration.

The nomenclature used to describe the number and location of double bonds in fatty acids can be found in a recent review (Cook, 1985) Due to the fact that rat liver phospholipids usually contain only one positional isomer of an unsaturated fatty acid I will use a shortened form of nomenclature that only gives information on the number of carbon atoms and double bonds. For example, to indicate that arachidonic acid is a twenty-carbon fatty acid containing four double bonds, I will use the abbreviation 20:4. Furthermore, a molecule of phosphatidylcholine containing palmitic acid and arachidonic acid will be referred to as 16:0-20:4-PC.

In biological membranes, the fatty acids are limited with respect to the *sn* position which they may occupy. Generally, saturated fatty acids are found at the *sn*-1 position while unsaturated fatty acids occupy the *sn*-2 position. However there are important exceptions to this rule. For example, in the retina there is a high proportion of PC molecules which contains 22:6 at both the *sn*-1 and *sn*-2 positions (Aveldano et al. 1977).

Phospholipids can also contain either an O-alkyl or an O-alkenyl ether species at the *sn*-1 position, instead of the more common acyl ester linkage. O-alkenyl ether lipids (plasmalogens) appear to be ubiquitous and have been described in mammalian, avian, molluscan, protozoan, bacterial and even plants systems (reviewed in Snyder, 1985) Generally, O-alkenyl moieties are found in ethanolamine-containing phospholipids while O-alkyl moieties are associated with choline-containing phospholipids. More will be said about these species in Section II.F.

The head groups of phospholipids found in biological membranes are choline, ethanolamine, serine, glycerol, phosphatidylglycerol and myo-inositol which are components of PC, PE, PS, PG, cardiolipin and PI, respectively (see Fig. I.3.).

I.D. Function of Phospholipids

I.D.I. Structural

In addition to providing a physical barrier the membrane phospholipids can provide a support matrix that cellular proteins may become attached to, via either noncovalent or covalent linkages

Noncovalent association of proteins with membranes is usually through either direct hydrophobic/ionic interactions with proteins or through indirect hydrophobic/ionic interactions, mediated by fatty acyl groups covalently attached to specific proteins (Schultz et al., 1988). The covalent association of proteins with membrane phospholipids is well documented. In the central nervous system there are a large variety of glycoproteins which are attached to the cell surface via covalent linkages to glycosylphosphatidylinositol. The role of PI, as an anchor for membrane associated proteins has recently been reviewed (Low, 1989).

I.D.II. Nonstructural

Signal Transduction

Currently signal transduction via the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) and other polyphosphinositides is considered to be one of the most important mechanisms by which external information is communicated to the cell (reviewed in Downes, 1990; Shears, 1989; Kikkawa et al., 1989). Briefly, a hormone, a growth factor or some other agent will induce, usually via the binding to a cell surface receptor, the hydrolysis of PIP_2 by a PI-specific phospholipase C. The hydrolysis of PIP_2 releases two biologically active compounds, diacylglycerol (DG) and inositol-3-phosphate (IP_3). IP_3 can activate the release of intracellular Ca^{2+} stores while DG can stimulate a Ca^{2+}-activated, phospholipid dependent protein kinase (PKC) (reviewed in: Exton, 1988; Loffelholz, 1989)

The exact source of cell DG, generated during signal transduction, is not clear. Besides PI, other membrane phospholipids could potentially contribute the DG molecule. Recently, PC (Pelech & Vance, 1989; Billah and Anthes, 1990; Exton, 1990) and phosphatidic acid (PA) (Loffelholz, 1989) have been suggested as precursors for the DG formed during cell activation. While PE is a major component of the cytosolic leaflet of the plasma membrane, the role of PE in signal transduction has been essentially ignored.

In addition to these two classical 'second messengers' there is a growing list of other membrane components which appear to be involved in the signal transduction pathway. For example, PA may interact with its own receptors and induce its own series of signals (Murayama et al., 1987). Sphingosine is thought to have a modulating role on the signal transduction pathway by both stimulating phospholipase D, thereby generating PA (Lavie et al., 1990), and by inhibiting protein kinase C (reviewed in: Zhang et al. 1990). Recently, Moolenaar et al. (1991) have presented evidence that suggest that even lysoPA may function as a mitogenic agent.

In summary, there are a great number of lipid compounds that could potentially participate in signal transduction pathways, and in addition to the classical 2nd messengers (DG and PIP_3) there is a growing list of alternate 2nd messengers that may modulate or transmit the incoming signal.

Activators/Conformation Stabilizers

There is a large volume of literature describing the ability of membrane lipids to activate or stabilize the activity of enzymes, receptors and ion channels. The following is a brief overview of this literature.

It is now widely accepted that lipids are directly involved in the unique regulation of two important enzymes of cellular lipid metabolism. The first of these enzymes, CTP: phosphocholine cytidylyltransferase (CT), can be the rate limiting enzyme in PC biosynthesis (see Section II.B.). The role of enzyme translocation in the regulation of CT was first proposed by Pelech and Vance in 1984 (Pelech and Vance, 1984). Presently, it is accepted that fatty acids and DG's cause the translocation of the enzyme from its cytosolic form (low activity) to the membrane associated form (high activity) (reviewed in: Vance, D. E., 1989). Furthermore, Jamil et al. have demonstrated that an increase in the amount of PC present in the endoplasmic reticulum will result in a decrease in the activity of membrane associated CT, presumably by causing the translocation of the enzyme from the membrane (Jamil et al., 1990).

The second enzyme, phosphatidate phosphohydrolase (PAP), regulates the formation of DG from PA and thereby controls the flux of precursors destined for the synthesis of

PC, PE and triacylglcerols (TG) (see Section II.A.). The translocation of this enzyme is regulated by fatty acids, in the form of acyl-CoA esters, and PA (reviewed in: Brindley, 1985a). Recently, Jamal et al. (1991)have suggested that the activity of a plasma membrane associated form of PAP may also be modulated by sphingosine.

Phospholipids have been shown, in a number of systems, to be a necessary component for the full expression of enzymatic activity. For example, Durieux et al. (1990) have shown, using Golgi vesicles isolated from porcine submaxillary ganglia, that the removal of PL completely abolished the activity of the enzyme UDP-glucose:ceramide glucosyltransferase. Enzymatic activity could be recovered with the addition of exogenous phospholipids. Phospholipids have been shown to modulate both the adenylate cyclase activity of rat liver plasma membranes (Colard et al., 1981; Gidwitz et al., 1980), and the binding of oxytocin to its receptor (Warr and Branda, 1983).

A specific function for the 18:0-22:6-PS molecular species of PS has been suggested by Abood et al. (1978). These authors have shown, using neural membranes and very low concentrations PLA_2 from bee venom, a system which they claim favors the preferential hydrolysis of the docosahexaenoic-rich fraction of PS, that opiate binding to its receptor is greatly inhibited even after a few percent of the total PS is hydrolyzed. These authors conclude that the PS closely associated with the receptor is the 18:0-22:6.

Fatty acids have also been shown to activate or modulate a wide variety of cellular enzymes. Fatty acids have been demonstrated to activate the membranous guanylate cyclase (Wallach and Pastan, 1976; Ignarro and Wood, 1987; Gerzer et al., 1986), purified preparations of protein kinase C (Seifert, 1988) and the insulin receptor tyrosine kinase-catalyzed phosphorylation of 422(aP2)protein (Hresko et al., 1990). Free fatty acids, possibly derived from phospholipids, have also been shown to be associated with the high molecular weight aminoacyl-tRNA synthetase complex and influence its structure and function (Sivaram and Deutscher, 1990). Fatty acids may also regulate ion channels. For example, it has been reported the arachidonic acid and other fatty acids can directly activate potassium channels in smooth muscle cells (Ordway et al., 1989).

Recently, it has been recently suggested that phospholipids may act as an energy source in the operation of the visual cycle (Deigner et al., 1989; Trehan et al.,1990). It has also been demonstrated that sea urchin spermatozoa utilize PC as a substrate for energy metabolism (Mita et al., 1988). Finally, membrane lipids have been implicated in bacterial DNA replication and in protein translocation across the inner membrane of gram-negative bacteria (reviewed in van den Boom and Cronan, 1989).

In summary membrane lipids can be implicated in an enormous number of cellular functions. While phospholipids are most commonly recognized as structural components of cell membranes and lipoproteins they are now being recognized as important players in signal transduction and other metabolic functions.

II. Biosynthesis of Phospholipids

The biosynthetic routes of all of the major phospholipid classes, in mammals, have recently been reviewed in great detail (Bishop and Bell, 1988; Vance, D. E., 1989; Tijburg et al. 1989). Thus, the following discussion will be be a brief overview on phospholipid biosynthesis in animals.

II.A. The de novo Biosynthesis of PA, DG, and CDP-DG

The routes for the de novo synthesis of PA, DG, and CDP-DG are depicted in Fig I.4. This pathway may be subdivided into three parts:

1) the reactions that lead to the formation of phosphatidic acid (PA).
2) the conversion of PA into DG, and
3) the conversion of PA into cytidine-5'-diphosphate-DG (CDP-DG).

The following section will review these three parts.

II.A.I. Biosynthesis of Phosphatidic acid (PA)

PA can be synthesized by three separate pathways in the rat liver:

1. from sn-glycerol-3-phosphate (G-3-P),
2. from dihydroxyacetone phosphate (DHAP), and
3. from DG.

In 1953, Kornberg and Pricer first demonstrated that palmitate and *sn*-glycerol-3-phosphate (G-3-P) were precursors of PA (Kornberg and Pricer, 1953). The first step in the synthesis of PA involves the acylation of G-3-P to 1-acyl-*sn*-glycerol-3-phosphate (lysoPA) and is catalyzed by the enzyme glycerol-3-phosphate acyltransferase (EC 2.3.1.15). This enzyme is found associated with the endoplasmic reticulum (Coleman and Bell, 1978) and the mitochondria (Shephard and Hübscher, 1969). The endoplasmic reticulum and the mitochondrial forms of this enzyme are distinct proteins (reviewed in Brindley and Sturton, 1982).

In 1968, Hajra and Agranoff demonstrated that DHAP was a precursor of PA (Hajra and Agranoff, 1968). In this pathway, LysoPA is formed by a two step process which involves the initial acylation of DHAP subsequently followed by the reduction of acyl-DHAP. The DHAP acyltransferase (EC 2.3.1.42) is found in both microsomal (Schlossman and Bell, 1977) and peroxisomal fractions (Hajra et al., 1979; Bates and Saggerson, 1979). The microsomal enzyme is the same enzyme which catalyses the acylation of G-3-P (Schlossman and Bell, 1977). The peroxisomal enzyme is a separate enzyme which can not acylate G-3-P (Hajra et al., 1979; Bates and Saggerson, 1979). The next reaction is catalyzed by the enzyme acyl-DHAP reductase, which is present in both microsomal and mitochondrial fractions.

The newly made lysoPA can then be acylated by the microsomal enzyme 1-acylglycerol-3-phosphate acyltransferase (EC 2.3.1.-), which is distinct from the microsomal glycerol-3-phosphate acyltransferase (Yamashita et al., 1972). The exact contribution which DHAP and G-3-P make to total PA production in the liver is unclear. There is considerable debate on the contribution made by each of these pathways. This debate has been reviewed (Brindley and Sturton, 1982) and it would appear that both these pathways may, under certain experimental conditions, predominate.

PA may also be formed by the direct phosphorylation of DG, a reaction catalyzed by the ATP-dependent enzyme DG kinase (Kanoh and Åkesson, 1978). The contribution of this pathway to overall PA production in the liver is unknown, but in the erythrocyte it may be a major route (Hokin and Hokin, 1963).

II.A.II. Conversion of PA into DG

The formation of DG involves the removal of the phosphate group from the PA molecule, and is catalyzed by the enzyme phosphatidate phosphohydrolase (PAP; EC 3.1.3.4). In the liver, there are at least two forms of this enzyme, a cytosolic form (Brindley, 1985b) and a plasma membrane bound form (Jamal et al., 1991). The cytosolic form of the enzyme has a strong dependency on Mg^{2+} (Bowley et al., 1977), is inhibited by sulfhydryl group blocking reagents (Butterworth et al., 1984), and has been demonstrated to translocate from the cytosol and to the endoplasmic reticulum membrane, where it becomes metabolically active (Moler and Hough, 1982; reviewed in: Brindley, 1988). During fat mobilization the levels of fatty acids, acyl-CoA esters and membrane phosphatidate accumulate and stimulate the translocation of cytosolic PAP onto the membrane of the endoplasmic reticulum (Brindley, 1988). The levels of cytosolic PAP may be increased during long term exposure to hormones such as glucocorticoids (Jennings et al., 1981), glucagon (Pittner et al., 1985), and growth hormone (Pittner et al., 1986). Interestingly, insulin was able to antagonize these effects (reviewed in: Brindley 1988). Little is known about the substrate specificity of this enzyme. In summary, the cytosolic PAP is a highly regulated enzyme which sits at the branch point of triacylglycerol and phospholipid biosynthesis. By regulating the cellular levels of PA, DG, and CDP-DG, PAP can control the rates of synthesis of most major lipids.

The plasma membrane PAP is a distinct protein which shows no Mg^{2+}-dependency, is not inhibited by sulfhydryl group blocking reagents, and is not regulated by the above mentioned hormones (Jamal et al., 1991). Because of its location and lack of regulation it is unlikely that this form of PAP is involved in triacylglycerol and phospholipid biosynthesis. It has been suggested that this enzyme is involved in modulating signal transduction pathways by controling the levels of PA in the plasma membrane (Jamal et al., 1991).

II.A.III. Conversion of PA into CDP-DG

PA can react with a molecule of the nucleotide cytidine-5'-triphosphate (CTP) and form cytidine-5'-diphosphodiacylglycerol (CDP-DG). This reaction is catalyzed by the enzyme PA: cytidylyltransferase (PACT: EC 2.7.7.41), which is located in both the mitochondrial (Petzold and Agranoff, 1967) and microsomal (Carter and Kennedy, 1966) fraction of cells. Very little is known about the physiological regulation of this enzyme. In vitro studies have shown that GTP can stimulate PACT activity while ATP and F^- can inhibit enzyme activity (Sribney et al., 1977). The 'activated' DG can then be utilized for the biosynthesis of phosphatidylglycerol and cardiolipin.

II.B. PC Biosynthesis

Phosphatidylcholine can be synthesized in mammalian tissue by at least five separate pathways. The routes for PC biosynthesis are:

1) The CDP-choline pathway,
2) the methylation pathway,
3) the reacylation of lysoPC.
4) the lysoPC: lysoPC pathway, and
5) the base exchange pathway

The first four of these pathways are outlined in Fig. I.5. The following section will cover the CDP-choline pathway, the methylation pathway, and the base exchange pathway. The pathways for reacylation of lysoPC will be covered in section III. B.

II.B.I. CDP-choline Pathway

The enzymatic steps of the CDP-choline pathway were elucidated by Kennedy et al. during the mid fifties (Kennedy and Weiss, 1956; Borkenhagen and Kennedy, 1957), and as such this pathway is commonly referred to as the Kennedy pathway. This pathway consists of the following three enzymes:

1) Choline kinase (CK),
2) CTP:Phosphocholine cytidylyltransferase (CT), and
3) CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT).

Choline kinase

CK (EC 2.7.1.32) is a cytosolic protein, which was first described nearly forty years ago by Wittenberg and Kornberg (Wittenberg and Kornberg, 1953). Thirty-two years later CK was purified to homogeneity from rat kidney and shown to have a molecular weight of 42 kD (Ishidate et al., 1984). The presence of at least three distinct forms of CK in rat liver, which range in size from 95 to 195 kD, have been described (Tadokoro et al., 1985). The molecular characterization of these isoenzymes and a summary of the enzymatic properties of CKs have recently been reviewed (Ishidate, 1989). While the gene for CK from yeast has been cloned and sequenced

(Hoska et al., 1989) such studies remain to be done on the mammalian CK(s). While choline kinase is essentially an irreversible enzyme, and is considered to be the commited step in the CDP-pathway it is not usually considered the rate limiting step in PC biosynthesis (Vance, D. E., 1989)

CTP:Phosphocholine cytidylyltransferase

The second and rate limiting enzyme of this pathway, CT (EC 2.7.7.15), was first discovered in 1956 and catalyzes the formation of CDP-choline from CTP (Kennedy and Weiss, 1956). This enzyme is located both in the cytosol (low activity) and microsomes (high activity) of rat liver and was first purified to homogeneity in 1986 (Weinhold et al.,1986). This enzyme has a molecular weight of 45 kD (Feldman and Weinhold, 1987) and becomes associated with the microsomal membranes under a wide variety of conditions which stimulate PC biosynthesis. For example, the addition of fatty acids to cultured cells (Pelech et al., 1983) or an increase in microsomal DG levels (Utal et al., 1991) favor the association of CT with membranes and stimulates PC biosynthesis. Conversely, the phosphorylation of CT (Sanghera and Vance, 1989) or the accumulation of PC in microsomal membranes (Jamil and Vance, 1990) will causes translocation of CT from the microsomes to the cytosol. The translocation of CT has been shown to be a physiologically important mechanism for controlling PC biosynthesis (Yao et el., 1990). Recently, the cDNA for rat liver CT was cloned and sequenced by Kalmer et al. (1990).

CDP-choline:1,2-diacylglycerol cholinephosphotransferase

The final reaction step involves the transfer of the phosphocholine constituent of CDP-choline to 1,2-diacylglycerol. This reversible reaction is catalyzed by the enzyme CPT (EC 2.7.8.2), an intrinsic membrane protein that has yet to be purified to homogeneity. CPT does not show any selectivity for diacylglycerol substrates (Ide and Weihold, 1982)

II.B.II. PE-methylation Pathway

In mammals there exists a second pathway for the synthesis of PC which appears to be significant only in liver (Ridgway, 1989a). This pathway involves a step-wise methylation of PE to form PC, which is catalyzed by the enzyme, phosphatidylethanolamine-N-methyltransferase (PEMT, EC 2.1.1.17). PEMT was first purified to homogeneity by Ridgway and Vance (1987) from rat liver microsomes and shown to have a molecular weight of 18.3 kD. This intrinsic microsomal protein is subject to regulation by PE and the ratio of *S*-adenosyl-L-methionine to *S*-adenosyl-L-homocysteine (Ridgway, 1989a). Using either microsomes or purified enzyme Ridgway and Vance (1988) have demonstrated that PEMT shows little substrate selectivity for PE molecular species.

The conversion of PE to PC is the only known pathway for the synthesis of choline in mammals. However, it has been suggested that choline could be formed, in the rat, by the direct methylation of ethanolamine. Recently, Andriamampandry et al. (1989) have demonstrated that the cytosol, isolated from primary cultures of neurons, will preferentially methylate phosphoethanolamine but not PE to choline-containing compounds. This methyltransferase activity was located in the cytosol but not in the microsomes indicating it was a distinct enzyme from PEMT. The significance of this finding is uncertain as PE methylation was the major route of choline biosynthesis in intact cells.

II.B.III. Base-exchange Pathway

The base exchange enzymes catalyze the incorporation of choline, ethanolamine and serine into pre-existing phospholipids (Kanfer, 1989). It is widely accepted that the primary route of serine incorporation into PS in animal cells is by the base exchange pathway (Hübscher, 1959). The enzyme which catalyzes the incorporation of serine into PS is called PS synthase and is found in the microsomal fraction (Dennis and Kennedy, 1972). While it is thought that the majority of serine incorporation into PS involves an exchange with PE, PC has also been shown to be an excellent substrate (Voelker, 1984; Miller and Kent, 1986; Kuge et al., 1986). This enzyme has been shown to be present on the cytosolic surface of liver microsomes (Bell, 1981). Muri and Kanfer (1976) have succeeded in chromatographically separating the individual activities of base exchange enzymes, which demonstrate selectivity for choline, ethanolamine and serine, from rat brain. An ethanolamine-serine base exchange enzyme has been purified, from rat brain microsomes, to homogeneity by Suzuki and Kanfer (1985) and shown to have a molecular weight of 100 kD. Ethanolamine and serine compete as substrates for this enzyme in vitro (Suzuki and Kanfer, 1985). Little is known about its regulation in vivo. While the base exchange pathway is important for PS synthesis the contribution which the base-exchange pathway makes to PC or PE biosynthesis is probably negligible. Kanfer (1980) has speculated that the choline and ethanolamine base-exchange enzymes may be more important in maintaining local membrane composition.

Overall regulation of PC biosynthesis

There are several experimental observations that indicate there is coordinate regulation between the CDP-choline and methylation pathways in the rat liver. It has been demonstarted that fatty acids will stimulate the CDP-choline pathway while inhibiting the methyltransferase pathway (Pelech et al., 1983; Audubert et al. 1984). Pritchard et al. (1982) have shown that inhibition of the methylation pathway will result in the stimulation of the CDP-choline pathway. Finally the inhibition of the CDP-choline pathway results in a stimulation of the methyltransferase pathway (Schneider et al., 1978).

PC biosynthesis may also be regulated by the topological arrangement of the enzymes on the endoplasmic reticulum. There is evidence which indicates that many enzymes of specific metabolic pathways are complexed in a sequential, nonrandom fashion (Srere, 1987). Recently, the channeling of intermediates through the CDP-choline pathway in cultured rat glioma cells has been demonstrated (George et al., 1989). These authors have suggested that the enzymes of the Kennedy pathway may be functionally linked, as a metabalon. Because of the importance of PC biosynthesis to the cell there probably exists many mechanisms by which cell PC levels are maintained. The elucidation of these mechanisms remain incomplete.

II.C. PE Biosynthesis

Phosphatidylethanolamine can be synthesized in mammalian tissue by at least four separate pathways. The routes for PE biosynthesis are:

1) The CDP-ethanolamine pathway,
2) the PS decarboxylation pathway,
3) the reacylation of lysoPE, and
4) the base-exchange pathway.

The first three of these pathways are outlined in Fig. I.6. The following section will cover the CDP-ethanolamine pathway, the PS decarboxylation pathway, and the base-exchange pathway. The reacylation of lysoPE will be covered in section III. B.

II.C.I. CDP-ethanolamine Pathway

The CDP-ethanolamine pathway is identical to the CDP-choline pathway. In the first step of this pathway ethanolamine is phosphorylated by the ATP-dependent ethanolamine kinase (EK, EC 2.7.1.32). Evidence has recently been summarized that indicates that CK and EK activities are located on the same protein (Ishidate, 1989).

The second and rate limiting enzyme of this pathway, CTP:phosphoethanolamine cytidylyltransferase (ET, EC 2.7.7.14), catalyzes the formation of CDP-ethanolamine from CTP and phosphoethanolamine. This enzyme is distinct from CT and is located only in the cytosol (Borkenhagen and Kennedy, 1957). ET has been partially purified from rat liver cytosol and shown to be a 100 kd protein, which consists of two 50 kD dimers (Sundler, 1975). ET is considered to be the rate limiting enzyme in the CDP-ethanolamine pathway (Sundler and Åkesson, 1975). Unlike CT, little is known about the regulation of ET.

The final reaction involves the transfer of the phosphoethanolamine constituent of CDP-ethanolamine to 1,2-diacylglycerol. This reversible reaction is catalyzed by the enzyme CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT, EC 2.7.8.1), an intrinsic membrane protein which is distinct from CPT (Cornell, 1989). Mammalian EPT has yet to be purified to homogeneity. Unlike CPT, EPT does show a strong selectivity for diacylglycerol substrates containing 22:6 at the sn-2 position (Holub, 1978).

II.C.II. PS decarboxylation Pathway

In 1951 Arstein demonstrated that L-serine was a precursor for ethanolamine (Arstein, 1951). Subsequently it was shown that the serine moiety of PS and not free serine was the substrate for a decarboxlase (Wilson, 1960). Dennis and Kennedy have clearly demonstrated that this reaction is catalyzed by the mitochondrial enzyme, PS decarboxylase (EC 4.1.1.65; Dennis and Kennedy, 1972). PS decarboxylase remains to be purified and characterized. Little is known about its regulation.

II.C.III. Base-exchange pathway

As previously mention for PC biosynthesis, PE can potentially be made by the base-exchange pathway. See Section II.B.III

Overall regulation of PE biosynthesis

Although de novo synthesis by the CDP-ethanolamine pathway is considered the major if not the primary route for PE synthesis in mammalian tissue (Bell and Coleman, 1980; Zelinski and Choy, 1982), there are some studies which have suggested that PS decarboxylation could account for essentially all of the PE formed. Voelker has demonstrated that PS is the major precursor of PE in BHK-21 cells cultured in the absence of ethanolamine (Voelker, 1984). More recently, Miller and Kent (1986) have suggested that the decarboxylation pathway is the sole route for PE synthesis in Chinese Hamster ovary cell lines. On the other hand, Xu et al. (1991) have recently demonstrated that both of these pathways must be considered in cultured glioma cells. Xu and his colleagues have calculated that exogenous serine is only 2-5% as effective as ethanolamine as a precursor for PE synthesis. Thus, the contribution that either of these pathways make to PE synthesis is most likely to depend on the supplies of ethanolamine and serine in the cell. PE may also be synthesized by the base exchange pathway but the importance of this route in the formation of PE in the rat liver is currently unknown.

PS decarboxylation and catabolism of PE is usually considered to be the major source of ethanolamine in nature. However, ethanolaminephosphate can also be derived from the catabolism of dihydrosphingosine phosphate (Stoffel et al., 1968). The exact contribution of this pathway to PE biosynthesis is unknown and usually is neglected.

II.D. PS Biosynthesis

See Section II.B.III

II.E. 1-alkyl- and 1-alkenyl-Phospholipid Biosynthesis

Generally, 1-O-alkenyl moieties are found in ethanolamine-containing phospholipids while 1-O-alkyl moieties are associated with choline-containing phospholipids. Ethanolamine plasmalogens can account for between 30 and 50% of serum ethanolamine phospholipids in rat (Vance, J.E., 1990) and humans (Myhers et al., 1989). Interestingly, the levels of ethanolamine plasmalogens in liver are low even though the liver has a relatively high capacity for plasmalogen synthesis (Snyder,1972). Recently this enigma appears to have been resolved. It has been demonstrated that lipoproteins secreted from cultured rat hepatocytes contain significant amounts of ethanolamine plasmalogens and been suggested that the liver could produce plasmalogens for export into the plasma (Vance, J.E., 1990).

Choline plasmalogens are rarely found in mammals. The most accepted explanation for their absence is that the microsomal enzyme, 1-alkyl-1-acylglycerophosphethanolamine desaturase, which converts O-alkyl to O-alkenyl ether species, is active with ethanolamine but not choline alkyl ethers (Paltauf et al., 1973). However, choline plasmalogens have been found in significant levels in the hearts of some but not all mammals. For example, 30-40% of total choline-containing phospholipids in human, guinea pig and rabbit hearts are plasmalogens while it is almost undetectable in rat and hamster hearts (Arthur et al., 1985; Wientzek et al., 1987).

Where do the myocardial choline plasmalogens come from ? The myocardial form of CPT can utilize 1-alkenyl-2-acylglycerol as a substrate (Poulos et al., 1968) and a plasmalogen selective myocardial phospholipase C has been described (Wolf and Gross, 1985). Thus, it has been suggested that, in the myocardium, ethanolamine plasmalogens are precursors to choline plasmalogens

III. Remodeling of Phospholipids

Once a phospholipid molecule has been synthesized inside the cell it has a number of metabolic fates. Phospholipids may be actively secreted from certain cell types. For example, hepatocytes are constantly secreting phospholipids in the form of lipoproteins (Wu and Windmueller, 1979) and bile (Davis et al. 1983) while pneumocytes secrete large amounts of dipalmitoyl-PC as a component of surfactants (van Golde et al., 1988). Phospholipids may be degraded by phospholipases or, as previously discussed in section I.II., there may be extensive interclass conversions (eg: PS to PE and PE to PC). Another metabolic fate of phospholipids is that of intraclass conversion or remodeling (ie. the conversion of 16:0-22:6-PC to 18:0-20:4-PC).

Remodeling reactions are considered to be primarily involved in the establishment and maintenance of specific molecular species in biological membranes (Lands, 1965). The role of membranes in physiologic adaptation of organisms to their environment has recently been reviewed in great depth and it was concluded that acyl chain and molecular species restructuring in phospholipids are the most common adaptations to environmental change (Hazel and Williams, 1990). Remodeling reactions may also be important in the removal of oxidized fatty acids from membrane phospholipids (Sevanian et al., 1983; van Kuijk et al., 1987). Remodeling reactions are also important in regulating the cellular levels of lysophospholipids. Savard and Choy (1982) have estimated that, in the hamster heart, the reacylation of lysoPC might account for almost 14% of the total production of PC.

Remodeling of phospholipids may take place by either the classical deacylation-reacylation pathway (Lands, 1965) or by the more recently discovered transacylation pathways (Chilton et al.,1986; Chilton et al., 1987; MacDonald and Sprecher, 1989). Fig I.7. shows the theoretical pathways for molecular species remodeling.

Briefly, in the deacylation-reacylation pathway, the acyl groups at the *sn*-1 or *sn*-2 positions are cleaved from the glycerophospholipid by either phospholipases A_1 or A_2, respectively. The lysophospholipid may then be reacylated by an acyl-CoA: lysophospholipid acyltransferase. This pathway has a distinct requirement for ATP, Mg^{+2} and coenzyme A (CoA, Masuzawa et al., 1989). Competing with these acyltransferases are lysophospholipases which will degrade the lysophospholipid. The free fatty acids, which are released by the phospholipases, must be activated to acyl-CoA by the enzyme(s) acyl-CoA synthetase (or ligase) prior to re-incorporation.

The transacylation pathway consists of three major routes which do not require ATP for the transfer reactions:

1) The CoA-dependent transacylation pathway,
2) the CoA-independent pathway, and
3) the lysoPC:lysoPC acyltransferase.

The CoA-dependent pathway has been described in numerous cell types and is characterized by its dependency on CoA. As the acyl-CoA: lysophospholipid acyltransferase has been demonstrated to be reversible (Irvine and Dawson, 1979) and can lead to the formation of an acyl-CoA in the presence of CoA but absence of ATP, it is not clear if a specific transferase enzyme is involved in this reaction or if this reaction is simply the reversal of the acyl-CoA: lysophospholipid acyltransferase.

The CoA-independent pathway catalyzes the transfer of arachidonic acid from a donor phospholipid, usually a diacylglycerophosphocholine (Kramer et al., 1983; Koehler et al., 1989), to an acceptor lysophospholipid, usually either a 1-alkyl- or 1-alkenyl-phospholipid (Masuzawa et al., 1989) without cofactors.

The final system for reacylating lysophospholipids was first described in rat liver (Erbland and Marinetti, 1965). In this system, rat liver microsomes catalyze the transfer of an acyl group from lysoPC to an acceptor lysoPC to form PC. The enzyme which catalyzes this reaction, lysoPC: lysoPC transacylase, has been purified from rabbit (Gross et al., 1983) and bovine heart microsomes (Sanjanwala et al., 1989).

The contribution each of these systems makes towards reacylation of lysophospholipids and ultimately towards membrane phospholipid composition is unclear. However, a recent study by Masuzawa et al. (1989) have demonstrated that, in rat brain microsomes, the deacylation-reacylation pathway, the CoA-dependent transacylation pathway, and the CoA-independent transacylation pathway all contribute to the reacylation of all the major phospholipids. In other systems, such as the heart or platelet one particular reacylation pathway may be predominant. Thus, the overall composition of membrane phospholipids will reflect both the specificities and activities of the enzyme(s) involved in the deacylation and subsequent reacylation reactions. It is the scope of the following section to review and discuss the enzymes involved in the deacylation and reacylation of phospholipids. Several enzymes also participate in the remodeling reactions by either providing substrates (acyl-CoA synthetases) or by competing for substrates (lysophospholipases). These enzymes will also be covered.

III. A. Deacylation of Phospholipids

III. A.I. Phospholipase A_1

The phospholipases are a class of enzymes which are characterized by their ability to hydrolyze one or more ester bonds of a phospholipid molecule. Accordingly, all those enzymes which hydrolyze the fatty acid at the *sn*-1 position of a phospholipid molecule are termed the phospholipases A_1 (EC.3.1.1.32., PLA_1s). Many lipolytic enzymes have the ability to hydrolyze the *sn*-1 fatty acid and thus PLA_1 activity has been described in a wide range of animal tissue (eg. brain, heart, liver, lung, ovaries, pancreas, platelets and plasma). Waite (1987) has recently reviewed the properties of the different mammalian PLA_1s and some generalizations can be made. The group of cellular enzymes that display PLA_1 activity can be further classified on the basis of their pH optimum and their subcellular location. Typically, those enzymes with an acidic pH optimum are of lysosomal origin and are collectively referred to as lysosomal PLA_1s. Another group of enzymes with PLA_1 activity are known to have a basic pH optimum and are usually associated with the microsomal or plasma membrane fraction. A third group of enzymes with PLA_1 activity are known to have a basic pH optimum and are usually found in the cytosol. The following discussion will focus on the purification and characterization of enzymes from these three PLA_1 groups.

Lysosomal PLA_1

The PLA_1 activity associated with lysosomes has been described in a number of systems (Franson et al., 1971; Hostetler et al., 1982; Okazaki et al., 1977). Waite et al. (1981) were the first to describe the purification of PLA_1 from rat liver lysosomes. PLA_1 is a glycoprotein with five isoenzyme forms, which differ in pI, molecular weight and kinetic properties (Hostetler et al., 1982). These differences are thought to reflect differences in the extent of protein glycosylation (Waite, 1987). The size of the lysosomal PLA_1 ranges from 15 to 90 kD (Hostetler et al., 1982) or from 45 to 60 kD (Waite et al., 1981). Recently, Hostetler et al. (1986) describe the 7600-fold purification of a PLA_1 from the lysosomal fraction of rat kidney cortex. They showed that this enzyme is also a glycoprotein, which has an apparent molecular weight of 30 kD and a pH optimum of 3.7. Interestingly, this enzyme appears to be regulated by inhibitory proteins.

Studies on the substrate specificity of the lysosomal PLA_1 have indicated that the apparent substrate specificity was more a reflection of the physical structure of the substrate than the actual specificity of the enzyme's active site for the substrate. For example, Robinson and Waite (1983) have studied the interaction of partially pure rat liver lysosomal PLA_1 with substrates. They found that the hexagonal-II structure of PE aggregates represents a preferred physical form for catalysis by PLA_1, while the bilayer form is less readily attacked. Thus, it would appear that PLA_1 does not demonstrate a specificity for a particular substrate per se, but rather prefers its substrates in a non-bilayer arrangement. To date there are no reports on the acyl-chain selectivity of lysosomal PLA_1.

Plasma Membrane and Microsomal PLA$_1$

Less well characterized are the PLA$_1$s, which are usually found associated with the microsomes and the plasma membranes. Scherphof et al. (1966) were the first to show that rat liver microsomes were enriched in PLA$_1$ whereas mitochondria were enriched in PLA$_2$. Subsequently, it was demonstrated that the rat liver PLA$_1$ is associated with the Golgi complex (van Golde et al. 1971) and with plasma membranes (Waite and van Deenen, 1967; Newkirk and Waite, 1971; Weglicki et al., 1971; van Golde et al. 1971, Nachbaur et al., 1972; Weglicki et al., 1972; Newkirk and Waite, 1973), but not with the mitochondria (Waite and van Deenen, 1967).

Newkirk and Waite (1973) demonstrated that the PLA$_1$ from rat liver plasma membranes and microsomes co-eluted during Sepharose 4B chromatography and both had a similar Ca^{2+} requirement. They also showed that the PLA$_1$ from plasma membranes was least active towards PI, and 2-, 8-, and 60-fold more active towards PC, PS, and PE, respectively. Waite and Sisson (1973a) clearly demonstrated that rat liver plasma membranes was the source of heparin releasable PLA$_1$ in plasma. The membrane PLA$_1$ is thought to be involved in lipoprotein metabolism and is often referred to as hepatic lipase. This enzyme has been purified to homogeneity and shown to be a monomer with an apparent molecular weight of 57 kD (Kuusi et al., 1979; Jensen and Bensadoun, 1981).

The plasma membrane PLA$_1$ has been shown to be specific for PE in some studies (Nachbaur et al., 1972; Weglicki et al., 1972) and rather unspecific in other studies (Waite and Sisson, 1973). Furthermore, the enzyme appeared to catalyze the transacylation of the acyl group from the *sn*-1 position of glycerides to an acceptor monoglyceride to form diglyceride (Waite and Sisson, 1973b). In 1974, Waite and Sisson showed that the enzyme could only catalyze the transfer of acyl groups at the *sn*-1 position and further suggested that the enzyme contained separate binding sites for acyl donor and acyl acceptor molecules.

In summary, the plasma membrane PLA$_1$/hepatic lipase demonstrates a broad substrate specificity and possesses both phospholipase and transacylase activity. It is not certain if the differences in substrate specificity reflect variations in assay conditions or in differences in the isolation of plasma membrane. Due to the location of this enzyme on the exterior of the cell it seems unlikely that this enzyme is involved in phospholipid remodeling reactions.

It is well established that the rat liver microsomal PLA_1 is very active towards both endogenous and exogenous PE and is specific for the *sn*-1 position (Bjornstad, 1966; Scherphof et al., 1966b; Newkirk and Waite, 1973; van Golde et al. 1971). It is still not clear if the plasma membrane associated PLA_1 is the same enzyme as the microsomal PLA_1. The purification of microsomal PLA_1 has yet to be accomplished and thus the possibility that these are two separate enzymes still exists.

Holub (1982) failed to demonstrate any *in vitro* selectivity of rat liver microsomal PLA_1 towards exogenously labeled PE molecular species. In a later study, Pind et al. (1985) also failed to demonstrate any *in vitro* selectivity of rat liver microsomal PLA_1 towards endogenously labeled PE molecular species. Pind et al. showed that both newly synthesized and 'older' bulk PE were equally accessible to the endogenous PLA_1 in their experimental system.

Cytosolic PLA_1

The presence of a PLA_1 in the cytosol of rat liver has been known for at least 25 years (Waite and van Deenen, 1967) and has been described in many systems (Hirasawa et al.1981; Fauvel et al. 1981; Dawson et al. 1982). In 1978, Suzuki and Matsumoto described a cytosolic PLA_1 in rat liver that had a pH optimum of 3.6 and was thought to be distinct from the lysosomal PLA_1 (Suzuki and Matsumoto, 1978). Since then, a cytosolic PLA_1 activity has been described in rat brain (Doherty and Rowe, 1980), and rat liver (Dawson et al. 1983), and rat heart (Nalbone and Hostetler 1985). The brain and liver enzyme both require Ca^{2+} for optimal activity and have a pH optimum of 9.5. The molecular weights of these three enzymes have not yet been determined. Recently, Cao et al. (1987) have described the 167-fold purification of a PLA from hamster heart cytosol. This enzyme has an apparent molecular weight of 140 kD and exhibits both PLA_1 and PLA_2 activity. The enzyme appears to be composed of six identical 14 kD subunits. The relationship between this enzyme and the other PLA_1s is unclear.

In terms of substrate selectivity, the cytosolic PLA_1 is similar to the lysosomal PLA_1, in that it prefers substrates in nonbilayer-hexagonal II phase (Dawson et al. 1983). The relationship between the cytosolic PLA_1 and the membrane PLA_1 is not well established. In addition, very little is known about the acyl-chain specificity of the cytosolic PLA_1.

In summary, there are three groups of PLA_1s that could potentially participate in phospholipid remodeling reactions. While these enzymes are isolated in different subcellular fractions their exact intracellular distribution, with the exception of the lysosomal enzymes, is for the most part unkown. It is still not clear if the microsomal PLA_1 is truly an endoplasmic reticulum enzyme or simply a contaminant (ie. hepatic lipase associated with the plasma membranes). Because of these considerations it is not clear which of these enzymes participate in deacylation-reacylation mechanisms.

III. A.II. Phospholipase A_2

All those enzymes which hydrolyze the fatty acid at the *sn*-2 position of a phospholipid molecule are considered as phospholipase A_2s (EC.3.1.1.4., PLA_2s). As a group, PLA_2s are much better characterized than the PLA_1s. PLA_2s are widely distributed in mammalian tissue and have been reviewed in two recent books (Waite, 1987; Wong and Dennis, 1989). Until recently, the best characterized of the PLA_2s, with respect to structure and function, were those that are secreted from the cell (ie. in snake venom or as part of the pancreatic digestive juice). These "extracellular" PLA_2s have been classified according to the pattern of their disulfide bonds, which is reflected in the position of the cysteines in the primary structure. Mammalian group I PLA_2s resemble the PLA_2 in pancreatic juice while the group II enzymes resemble the PLA_2 in snake venom from the crotalid and viperid species (Wong and Dennis, 1989). In general, group I and II enzymes are small (M_r:12 to 15 kD), relatively stable and dependent on Ca^{2+} for optimal activity.

The "intracellular" PLA_2s are not as well understood, reflecting the low levels of these enzymes in cells and the subsequent difficulties associated with their purification (Ulevitch et al., 1988; Aarsman et al., 1989). Though present in very low levels, these PLA_2s are extremely important in phospholipid metabolism (eg. remodeling) and in the generation of precursors of biologically active lipids (eg. eicosanoids and platelet-activating factor) (Snyder, 1989). Thus, it became apparent that to understand fully the role of the "intracellular" PLA_2's these enzymes had to be purified and characterized.

Currently, "intracellular" PLA_2s have been purified and characterized from over fifteen different sources. There appears to be at least three groups of enzymes and they can be classified as follows:

1. Low molecular weight, Ca^{2+}-dependent enzymes,
2. High molecular weight, Ca^{2+}-dependent enzymes,

and 3. High molecular weight, Ca^{2+}-independent enzymes.

The following discussion will focus on the purification and characterization of these three groups of intracellular PLA_2s.

Low molecular weight, Ca^{2+}-dependent enzymes

The classic example of this first class of enzyme is the rat liver mitochondria PLA_2. It has long been recognized that rat liver mitochondria contain a PLA_2 (Waite, 1969). However, the physiological roles of this enzyme have not clearly been established even though the enzyme has been implicated in the catabolic part of phospholipid remodeling (Pfeiffer et al., 1979; Wiswedel et al., 1982). This enzyme was initially purified 160-fold from rat liver mitochondria (Waite and Sisson, 1971). Subsequently, it was purified to homogeneity and shown to be a stable 15 kD protein that was Ca^{2+}-dependent (de Winter et al., 1982). More recently, the immunoaffinity purification, partial sequence, and subcellular localization of the rat liver PLA_2 has been reported (Aarsman et al., 1989). Studies with a monoclonal antibody to this enzyme, demonstrated that the PLA_2 activity consisted almost exclusively of a single or closely related enzyme species. Furthermore, this species was predominantly located in the mitochondria. The sequence of the first 24 amino acids of the enzyme were determined and compared to other known sequences. This part of the sequence showed only 25% homology with that of rat pancreatic PLA_2 (Ono et al., 1984) but was 96% identical with that of rat platelet (Hayakawa et al., 1987) and rat spleen membrane-associated PLA_2 (Ono et al. , 1988).

Presently, non-pancreatic Ca^{2+}-dependent enzymes have been purified from a number of different sources including rat platelets (Hayakawa et al.,1988a), human platelets (Kramer et al., 1989), rat spleen membranes (Ono et al , 1988), rat lung (Lindahl et al., 1989), rabbit lung (Filgueiras and Possmayer, 1990), porcine intestine (Verger et al., 1982), rabbit ascites (Forst et al., 1986), human rheumatoid synovial fluid (Stefanski et al., 1986), rabbit polymorphonuclear cells

(Wright et al., 1990), and the macrophage-like cell line, P338D1 (Ulevitch et al., 1988). A comparison of protein sequences have indicated that the PLA_2 from rat platelets and rat spleen membranes are identical with the secreted form of PLA_2 from rat platelets (Hayakawa et al., 1988a). However, these three enzymes are only 59% homologous with the PLA_2 from rabbit ascitic fluid. It is not clear if the difference between the rat liver mitochondria PLA_2 and the rat platelet PLA_2 protein sequence, which appears to be the N-terminal amino acid only, represent two isoforms of the enzyme or are simply the result of sequencing errors.

Recently, work by Inada et al. (1991) have raised questions concerning the cellular origin of the rat liver mitochondria PLA_2. This group has used a polyclonal antibody, raised against PLA_2 from rat spleen membrane, to demonstrated that Kupffer cells contain 6-fold greater activity than hepatocytes. Furthermore, recent work by Wilton et al. (1991) suggests that the rat liver mitochondria PLA_2 is not actually present in the hepatocyte at all. Wilton et al. suggest that the "mitochondrial PLA_2" is derived from rat liver Kupffer cells and becomes associated with the mitochondrial fraction during isolation of the organelle. Beyond this controversy it is interesting to note that the rat spleen membrane PLA_2 has a presequence that is characteristic of a secretory protein (Inada et al., 1991) whereas the rat liver mitochondrial enzyme presumably has an extension sequence, absolutely necessary for transport into the mitochondria (Furuya et al., 1987). Thus, this issue should be resolved by sequence analysis of the cDNA for the rat liver mitochondrial enzyme.

Because of the putative role of PLA_2s in the generation of arachidonic acid destined for the biosynthesis of eicosanoids there have been a number of attempt at demonstrating that intracellular PLA_2s selectively hydrolyzes arachidonic acid. Purdon et al (1987) have shown that human platelet PLA_2 preferentially hydrolyzed 20:4-containing molecular species of PE and PC. They further suggested that a certain proportion of 20:4-containing molecular species of PE and PC are compartmentalized within the platelet membrane, proximal to the site of action of this enzyme. Ballou et al. (1986) showed that the rat platelet PLA_2 demonstrated specificity for arachidonic acid. Interestingly, they further suggested that there were two forms of PLA_2 in the rat platelet, one Ca^{2+}-dependent showing selectivity for PC and another Ca^{2+}-independent showing preference for PE. In light of the recent purification of a single PLA_2 from rat platelets (Hayakawa et al.,1988b) it would appear that the differences in specificity may be represent assay conditions rather than different forms of the enzyme. Recently, Schalkwijk et al. (1990) have studied the acyl-chain selectivities of PLA_2 derived from human PMN cells, human platelets and rat platelets using *sn*-2-[^{14}C]oleoyl-labeled PC and *sn*-2-[^{3}H]arachidonyl-labeled PC. No evidence for acyl-chain selectivity was observed for human PMN cells and rat platelets. Results with human platelet cytosol were highly suggestive for the presence of an 20:4-selective PLA_2 when separate PC species were assayed. This apparent selectivity was lost when the substrates were mixed or embedded in a membrane of 1-palmitoyl-2-linoleoyl-PC. There appears to be no consensus with regard to the substrate specificity of this group of phospholipases.

High molecular weight, Ca^{2+}-dependent enzymes

In the past five years a new group of high molecular weight, Ca^{2+}-dependent intracellular PLA_2's have been identified and purified from a number of different sources including sheep platelets (Loeb and Gross, 1986), rat kidney (Gronich et al., 1990), mouse peritoneal macrophages (Wijkander and Sundler, 1989), and two leukemic cell lines; RAW 264.7 (Leslie et al. 1988) and U937 (Diez and Mong, 1990; Clark et al., 1990). In general, these enzymes range in size from 30 to 110 kD, are Ca2+-dependent, and are relatively unstable.

Wijkander and Sundler (1989) reported the purification of a 70 Kd cytosolic PLA_2 from mouse peritoneal macrophages. This enzyme is active on PI, PE, and PC and shows selectivity for PC species containing 20:4 in the *sn*-2 position compared to PC species containing 18:1 in the *sn*-2 position. This enzyme appears to be distinct from the enzyme(s) purified from the leukemic cell lines RAW 264.7 and U937.

Several groups have described the purification of the large PLA_2 from leukemic cell lines. Leslie et al. (1988) first described the enzyme from the RAW 264.7 cell line as an arachidonyl-hydrolyzing selective PLA_2 with an approximate molecular weight of 60 kD. Subsequently, a PLA_2 with a molecular weight of 56 kD was described in the U937 cell line (Diez and Mong, 1990). Concurrently, another group reported that the PLA_2 from the U937 cell line had a molecular weight of 110 kD and that a major contaminant in the 60 kD molecular weight range was removed in their final purification step (Clark et al., 1990). Interestingly, Clark et al. used the same purification protocol to isolate a PLA_2 from the RAW 264.7 cell line, and reported that this enzyme had a molecular weight of 105 kD. This controversy was resolved when a fourth group demonstrated that the PLA_2 from U937 was about 100 kD protein

(Kramer et al., 1991). It is now accepted that the enzyme, isolated from both cell lines, is a 100 kD protein (Leslie, 1991) and probably is the same protein. It remains to be determined if the mouse peritoneal macrophage enzyme belongs to this group.

All these enzymes are active at physiological concentrations of Ca^{2+}. Recently, Channon and Leslie (1990) proposed a mechanism for the association of the PLA_2 with membranes in the macrophage cell line, RAW 264.7. They suggest that in an unstimulated cell the PLA_2 is found in the cytosol. Upon stimulation the cytosolic Ca^{2+} concentration increases, and the PLA_2 would translocate to the membrane and begin hydrolyzing arachidonyl-containing phospholipids. Consistent with this model is the demonstration that the U937/RAW 264.7 PLA_2 exhibits arachidonyl-hydrolyzing selectivity (Leslie et al., 1988; Clark et al., 1990; Diez and Mong, 1990).

PLA_2 activities that hydrolyze arachidonic acid have also been described in mouse peritoneal macrophages (Wightman et al., 1981) and in the mouse macrophage cell line, P388D1 (Ulevitch et al., 1988; Lister et al., 1988). In macrophages a relatively large portion of choline-containing phospholipids consisit of the 1-alkyl-linked species, which is considered to be the membrane precursor of the lipid mediator platelet activating factor (PAF) (Albert et al. 1983; Nakagawa et al.,1985). In the macrophage the 1-O-alkyl-linked species is highly enriched in arachidonic acid (Nakagawa et al. 1985). Thus, it has been proposed that the PLA_2 hydrolysis of 1-O-alkyl-linked PC would result in the coordinate production of PAF and eicosanoids (Albert et al.,1983; Nakagawa et al.,1985). A cellular PLA_2 has been implicated in the release of arachidonic acid from mouse peritoneal macrophages (Emilsson et al., 1984) and human neutrophils (Chilton et al., 1988).

Interestingly, all of these enzymes possess lysophospholipase activity. This activity would allow the enzyme to catabolize selectively the 1-acyl-lysophospholipids generated during arachidonic acid release while leaving the lysoPAF molecules, also formed during arachidonic acid release, available for acetylation to PAF.

High molecular weight, Ca^{2+}-independent enzymes

This group of PLA_2s have been isolated in only two types of tissue. A calcium-independent, plasmalogen-selective PLA_2 activity has been described in canine myocardial cytosol (Wolf and Gross, 1985). Recently, this enzyme has been purified and characterizated (Hazen et al., 1990). They reported that the enzyme has an apparent molecular weight of 40 kD. and that it demonstrates specificity for arachidonate-containing 1-O-alkenyl-PC.

A 96 kD PLA_2 from guinea pig intestinal brush-border cells has recently been described (Gassama-Diagne et al., 1989). This enzyme was initially shown to be tightly associated with brush-border membranes (Diagne et al., 1987). Subsequently the enzyme could be released from the membrane by digestion with papain. This observation has lead to the suggestion that this enzyme is composed of two domains, a small hydrophobic tail which functions to anchor the protein to the membrane and a larger domain containing the catalytic site (Gassama-Diagne et al., 1989). In terms of enzyme specificity, this enzyme is more active against diacylglycerophospholipids than against 1-O-alkyl-phospholipids. In addition this enzyme also possesses lysophospholipase activity.

In summary, there are three groups of PLA_2s that could potentially participate in remodeling reactions. While the exact in vivo specificities of these enzymes is not known, some in vitro data is available which suggests that phospholipids with a polyunsaturated fatty acid in the *sn*-2 position are preferentially hydrolyzed. However, the exact role, if any, which these enzymes play in remodeling reactions is at present unknown: clearly the potential is there.

Evaluation of PLA_2 in vitro substrate selectivity

Under certain assay conditions the same PLA_2 can be made to demonstrate selectivity for both phospholipid classes and molecular species, while under other conditions this enzyme will show little substrate selectivity. In many cases in vitro specificities may not reflect actual in vivo specificities. Since many of the enzymes involved in deacylation, reacylation and transacylation reactions must first find its substrate in a membrane before using it as a substrate, in vitro specificities may not reflect the interaction of the enzyme with the substrate but rather the interaction of the enzyme with the lipid environment. Obtaining data on in vivo specificities is very difficult and thus we must rely on in vitro specificities. Yet, how do we decide on what in vitro data to trust? This question has been addressed by a number of studies and is an important concept throughout this thesis. Thus, as an example, the following section will try and review the literature on PLA_2 substrate selectivities.

de Winter et al. (1987) have studied the hydrolysis of membrane-associated phospholipids by mitochondrial PLA_2. They reported that the hydrolysis of PE by mitochondrial PLA_2 proceeds nearly entirely via intramembrane enzyme action (the PLA_2 acts on substrate in its own membrane and not in other membranes). The rate of PE hydrolysis was about twice that for PC and about 10-fold that for cardiolipin. In an accompanying paper, Lenting et al. (1987) have found that the hydrolysis of exogenously added PE and PC is preceded by an association of the substrates with the membranes. PE associated more extensively with the biomembrane than PC, and this association was stimulated by Ca^{2+}. They concluded that the high preference for exogenous PE results in part from its physicochemical properties, allowing a better association with biomembranes, and does not reflect enzyme specificity per se. Thus, the physical presentation of the substrate to the enzyme clearly dictates the rate of hydrolysis, and assay conditions can favor the presentation of one substrate over another (pH, ionic strength, and Ca^{2+} concentrations)

As mentioned earlier, Schalkwijk et al (1990) have studied the acyl-chain selectivities of several PLA_2 preparations. Results with human platelet cytosol were highly suggestive for the presence of an arachidonyl-selective PLA_2 when separate PC species were assayed. This apparent selectivity was lost when the substrates were mixed or embedded in a membrane of 1-palmitoyl-2-linoleoyl-PC. However, the apparent specificity for arachidonic acid appears to be mainly caused by a K_m effect, which may be influenced by substrate organization rather than being a true acyl-chain specificity due to specific recognition of the arachidonate moiety in the active site of the enzyme. Membrane density can be decreased by increasing the degree of unsaturation in the *sn*-2 fatty acid of PC (Demel et al., 1972) and since membrane packing can dramatically affect the penetration of a PLA_2 into the membrane (Demel et al., 1975), experiments which use substrate vesicles of homogeneous composition to determine enzyme specificity should be viewed with caution (Schalkwijk et al., 1990).

Another source of error when determining substrate specificities has become apparent from the work of Diez et al. (1990). They have demonstrated that differences in *in vitro* substrate specificity reported for PLA_2 activities located in distinct subcellular particulates may be a reflection of the distinct phospholipid and fatty acid composition in these particulates (Diez and Mong, 1990).

In summary, it appears that the composition and physical state of the substrate must be carefully considered when determining substrate specificities of intracellular PLA_2's by the use of both endogenous and exogenous substrates

III. A.III. Lysophospholipase

Lysophospholipases (EC 3.1.4.39) are probably as widely distributed as any PLA_1 or PLA_2. This group is only mentioned in this discussion because it can compete directly with the enzymes of the reacylation pathways for the lysophospholipid substrate thereby affecting membrane phospholipid composition. These enzymes are usually neglected during discussions on remodeling reactions but clearly can play a deciding role on the final fate of a lysophospholipid.

de Jong et al. (1974) purified two lysophospholipases from beef heart, a 65 and a 25 kD protein, and showed that these enzymes lacked substrate specificity. More recently, Zhang and Dennis (1988) purified and characterized a two lysophospholipases from the macrophage-like cell line p388D1. These two enzymes had an apparent molecular weight of 27 and 28 kD and did not possess PLA_1, PLA_2 or lysosphospholipase-transacylase activity. For a more in depth examination of this group of phospholipases the reader is directed to a recent book (Waite, 1987).

III. B. Reacylation of Phospholipids

III. B.I. Acyl CoA synthases

Long-chain fatty acids are converted to their 'activated' acyl-CoA forms by the enzyme(s) non-specific acyl-coenzyme A synthetases (EC 6.2.1.3., Acyl-CoA synthetases). While these enzymes do not have a direct role in the reacylation of lysophospholipids they are able to regulate the availability of acyl-CoAs in the cell and thus, like the lysophospholipases, influence the fate of the lysophospholipids. These enzymes play a central role in the metabolism of lipids in the cell. Tomoda et al. (1991) has recently demonstrated that the inhibition of non-specific acyl-CoA synthetase leads to an inhibition of lipid synthesis and cell proliferation. Non-specific acyl-CoA synthetase has been isolated and purified from the microsomal and mitochondrial fractions of rat liver (Tanaka et al., 1979). These enzymes are identical with respect to molecular weights (approx. 76 kD), amino acid composition and substrate specificity. Recently, Suzuki et al. (1990) has cloned and sequenced the cDNA for this enzyme and shown it to correspond to a protein of 78.2 kD.

However this story is far from over as there appears to be more than one acyl-CoA synthetase. Philipp and Parson (1979) have reported very different non-specific acyl-CoA synthetases in rat liver microsomal and mitochondria fractions. Recently, the presence of at least two acyl-CoA synthetases in the rat liver microsomes and peroxisomes has been reported (Lageweg et al., 1991). In addition, there have been several reports of an arachidonyl-CoA specific acyl-CoA synthetase in the microsomes of platelets (Laposata et al., 1985) and brain (Reddy and Bazan, 1983). The existence of an arachidonyl-CoA specific acyl-CoA synthetase is further supported by studies on the cell line $HSDM_1C_1$, derived from a fibrosarcoma. $HSDM_1C_1$ is defective in the incorporation of arachidonic acid into cellular phospholipids and the defect has been shown to be in the arachidonyl-CoA specific acyl-CoA synthetase (Neufeld et al., 1984). Furthermore, Laposata et al. (1985) have clearly demonstrated that $HSDM_1C_1$ is not defective in the non-specific acyl-CoA synthetase activity. Kinetic studies on the arachidonyl specific acyl-CoA synthetase have shown that while arachidonic acid is a preferred substrate docosahexaenoic acid is also a good substrate (Reddy and Bazan, 1983).

Thus, unlike the lysophospholipases, there is good evidence for specific acyl-CoA synthetases being involved in the preferential activation of certain fatty acids that, in the case of arachidonic acid and docosahexaenoic acid, are destined for the reacylation pathway.

III. B.II. Acyl-CoA: lysophospholipid acyltransferase

The second part of the deacylation-reacylation pathway involves the reacylation of the lysophospholipid with an acyl-CoA. Depending on both the initial phospholipid substrate and the nature of the phospholipase, the lysophospholipid intermediate can be either a 1-acyl-, 1-alkyl-, 1-alkenyl, or a 2-acyl-lysophospholipid. Potentially, the subsequent reacylation could be accomplished by a single acyl-CoA:lysophospholipid acyltransferase (EC 2.3.1.23., ACLAT). However, this does not appear to be the case as guinea-pig heart has been demonstrated to have distinct acyl-CoA: 1-acyl-, 1-alkyl-, 2-acyl-lysophospholipid acyltransferases (Arthur and Choy, 1986; Arthur et al., 1987a; Arthur, 1989).

ACLATs in general, are usually localized in the microsomal fraction of most tissue (Severson and Fletcher, 1985; Gross and Sobel, 1982) but have been demonstrated in the mitochondrial fraction as well (Arthur and Choy, 1986; Arthur et al., 1987a). Recently, Sanjanwala et al. (1988) has described the purification of an acyl-CoA: 1-acyl-*sn*-glycerophosphocholine acyltransferase from bovine heart muscle. This enzyme was shown to be a 65 kD protein that displayed little substrate specificity with respect to lysophospholipid acceptor molecules. Microsomal acyl-CoA: 1-acyl-lysophospholipid acyltransferases have been studied in great detail, with respect to selectivity for acyl-CoAs, in various tissues (Lands and Hart, 1965; Lands et al., 1982; Okuyama et al., 1983; Arthur and Choy, 1986; Arthur et al., 1987b; Baker and Thompson, 1973; Waku and Lands, 1968; McKean et al., 1982; Inoue et al., 1984; Waku and Nakazawa, 1977; Masuzawa et al., 1986) and in general exhibits a preference for polyunsaturated fatty acyl-CoAs but not the acceptor molecule.

Acyl CoA: 2-acyl-lysophospholipid acyltransferases have not been studied in great detail. Early studies suggested that these enzymes had a great selectivity for saturated fatty acyl-CoAs (Lands and Merkle , 1963; van den Bosch et al., 1968; Holub, 1981). Arthur (1989) has recently re-examined this question and has shown that, in the guinea-pig heart, this enzyme does not demonstrate any specificity for acyl-CoAs. However, he did show that the enzyme prefers 2-arachidonyl-LPC over 2-palmitoyl-LPC as an acceptor.

As mentioned earlier, the acyl-CoA: lysophospholipid acyltransferase has been demonstrated to be reversible (Irvine and Dawson, 1979). One explanation for the presence of a CoA-dependent transacylation pathway is that this enzyme is working in reverse as a transacylase. However, while Sugiura et al. (1988) have recently presented evidence that suggests that the CoA-dependent transacylase is not the acyl-CoA: lysophospholipid acyltransferase working in reverse, there is no direct evidence for the existence of a separate transacylase. Thus, the CoA-dependent transacylation pathway will be covered under this section and not a separate section.

The CoA-dependent transacylation pathway has been described in many systems including: human neutrophils (MacDonald and Sprecher, 1989), rat brain and liver microsomes (Ojima et al., 1987; Sugiura et al., 1988), dog heart microsomes (Reddy and Schmid, 1987), and platelets (Kramer et al., 1984a, Breton et al., 1988). This enzyme appears to have a wide range of both donor and acceptor substrates specificities, depending on the tissue being studied. While T-lymphocytes specifically transfer arachidonate from PC to plasmalogen PE (Trotter et al., 1982), murine and rabbit macrophage catalyze the transfer of arachidonate from PI to 1-alkyl-PC and 1-alkenyl-PE, from PC to 1-alkenyl-PE and from PE to 1-alkyl-PC (Robinson et al., 1985; Sugiura et al., 1987a). In macrophages the CoA-dependent transacylation pathway is not specific for arachidonate transfer and in fact demonstrates a lack of specificity for most fatty acids (Flesch et al., 1984).

In summary, there remains a number of unanswered questions concerning the Acyl-CoA: lysophospholipid acyltransferases. How many are present within a cell? What are the substrate specificities of these individual enzymes? And finally, what role do these enzymes play in the in vivo remodeling processes? It is obvious that they do play a role in phospholipid remodeling but is this role an active or passive one?

III. B.III. CoA-Independent Transacylases

CoA-Independent transacylation is unique in that the transfer of acyl groups between phospholipids does not appear to require cofactors and no free fatty acid is generated during the reaction. CoA-Independent transacylation pathway has been described in many systems including: human platelets (Kramer and Deykin, 1983), rat platelets (Colard et al., 1984), human neutrophils (Chilton et al., 1987; Chilton et al., 1986; MacDonald and Sprecher, 1989), and macrophages (Trotter et al., 1982; Flesch et al., 1984). This pathway involves the transfer of an acyl group from a donor phospholipid, usually a diacylglycerophosphocholine, to an acceptor lysophospholipid, usually either a 1-alkyl- or 1-alkenyl-phospholipid. For example, arachidonic acid is transferred from 1,2-diacylglycerolphosphocholine to 1-alkenyl-lysoPE in human platelets (Kramer and Deykin, 1983) and to 1-alkenyl-lysoPE and 1-alkyl-lysoPC in macrophages (Sugiura et al., 1984; Sugiura et al., 1985; Sugiura et al., 1987b, Robinson et al., 1985).

The fatty acid being transferred is usually arachidonate or docosahexaenoate (Koehler et al., 1989; Chilton et al., 1983). Recently, Sugiura et al. (1987a) examined the donor and acceptor selectivities of the CoA-independent transacylase in rabbit alveolar macrophages. They found that 20:4 , 20:5 (n-3), 22:4(n-6), and 22:6(n-3) are all transferred to lyso-PAF at the same rate. Because of the selective transfer of arachidonate this pathway has become a focal point of arachidonic acid metabolism research.

Thus, in contrast to the CoA-dependent transacylation pathway, this pathway appears to be a specific route for the incorporation of polyunsaturated fatty acids into alkyl- and alkenyl-linked phospholipids.

III. B.IV. LysoPC: LysoPC transacylase

LysoPC: LysoPC transacylase (EC 3.1.1.5) catalyzes the transacylation of a fatty acid from one molecule of lysoPC to another molecule of lysoPC. The products of this reaction are PC and glycerophosphocholine. Erbland and Marinetti (1965) first described this activity in rat liver. Subsequently, this enzyme has been reported in erythrocytes, leukocytes, rabbit and mouse lung, human plasma and intestinal and gastric mucosa (van den Bosch et al., 1981). This enzyme has been purified from the cytosolic fraction from rat lung (van den Bosch et al., 1981), rabbit lung (Casals et al., 1982), and rabbit myocardium (Gross et al., 1983). The enzymes from rat lung and rabbit myocardium have been well characterized. These enzymes have different molecular weights (78 kD and 63 kD, respectively) behave differently towards substrates and thus appear to be unrelated. More, recently, Sanjanwala et al. (1989) have described the purification of a bovine heart microsomal enzyme which catalyzes the same reaction as the above cytosolic enzymes. This enzyme has a molecular weight of 128 kD.and only catalyzes the exchange of fatty acids between lysoPC molecules.

IV. Rationale and Objectives of Thesis

It is apparent from the above discussion that the phospholipid composition of a cell membrane is dictated by the action of both biosynthetic and degradative enzymes. The activities of these enzymes are in turn modulated by a number of different factors including hormones, diet, and age. The liver is unique in the body, in that it possesses at least five separate pathways for the formation of phosphatidylcholine and at least four separate pathways for the formation of phosphatidylethanolamine, which have been shown to provide distinct pools of phospholipids inside the hepatocyte. The routes of phospholipid biosynthesis have been the major focus of our lab for many years. More recently, we have become interested in the dynamics of the newly-made phospholipids. It was the purpose of this study to examine the fate of newly-made phospholipids at the molecular level. By using high-pressure liquid chromatography to examine the individual molecular species of phospholipids we hoped to gain new insights into the metabolic fates of phospholipids synthesized in the hepatocyte. The experiments in this study were designed with the following aims:

1) To study the fate of newly made [^{3}H]ethanolamine-labeled PE and PC inside normal rat hepatocytes.

2) To study the fate of newly made [^{3}H]ethanolamine-labeled PE and PC inside normal rat hepatocytes during prolonged inhibition of the enzyme PEMT.

3) To study the fate of newly made [^{3}H]serine-labeled PE and PC inside normal rat hepatocytes.

4) To study the fate of newly made [^{3}H]choline-labeled PC inside normal and choline deficient rat hepatocytes.

5) To study the catabolism of [^{3}H]ethanolamine-labeled PE in rat liver microsomes and mitochondria.

FIGURE I.1.

PHOSPHOLIPID COMPOSITION OF RAT LIVER MEMBRANES

This diagram depicts the phospholipid composition of different membranes isolated from rat liver. Phosholipids are: sphingomyelin; cardiolipin; lysophospholipids, LysoPL; phosphatidic acid, PA; phosphatidylserine, PS; phosphatidylinositol, PI; phosphatidylcholine, PC; and phosphatidylethanolamine, PE. (data taken from Daum, 1985).

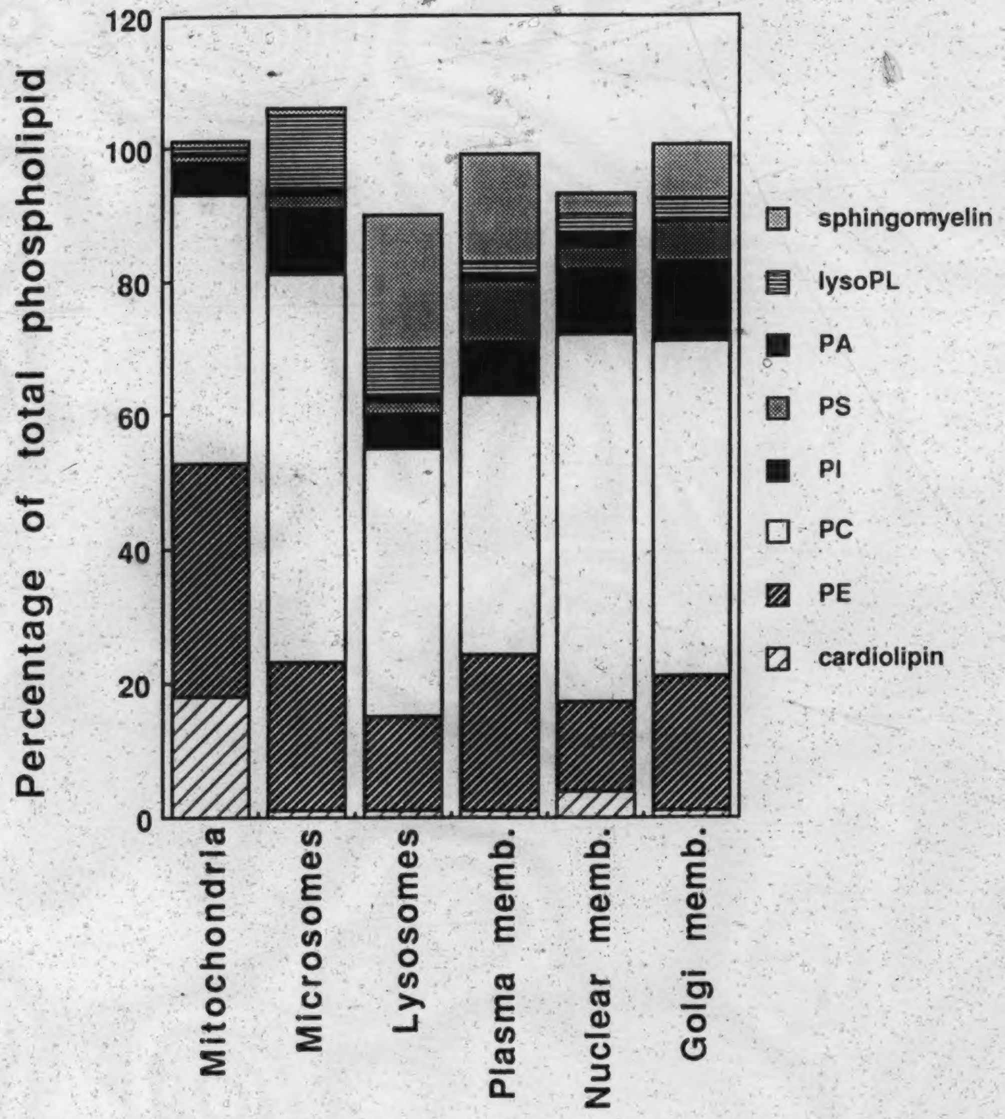

Percentage of total phospholipid
120
100
80
60
40
20
0
Mitochondria
Microsomes
Lysosomes
Plasma memb.
Nuclear memb.
Golgi memb.
sphingomyelin
lysoPL
PA
PS
PI
PC
PE
cardiolipin

FIGURE I.2.

THE FLUID MOSAIC MODEL

This cartoon illustrates the central features of the 'fluid mosaic' model (Singer and Nicholson, 1972). Two leaflets of phospholipid are shown forming a bilayer structure. Proteins (P) are shown interacting with either the inner leaflet or with the entire membrane.

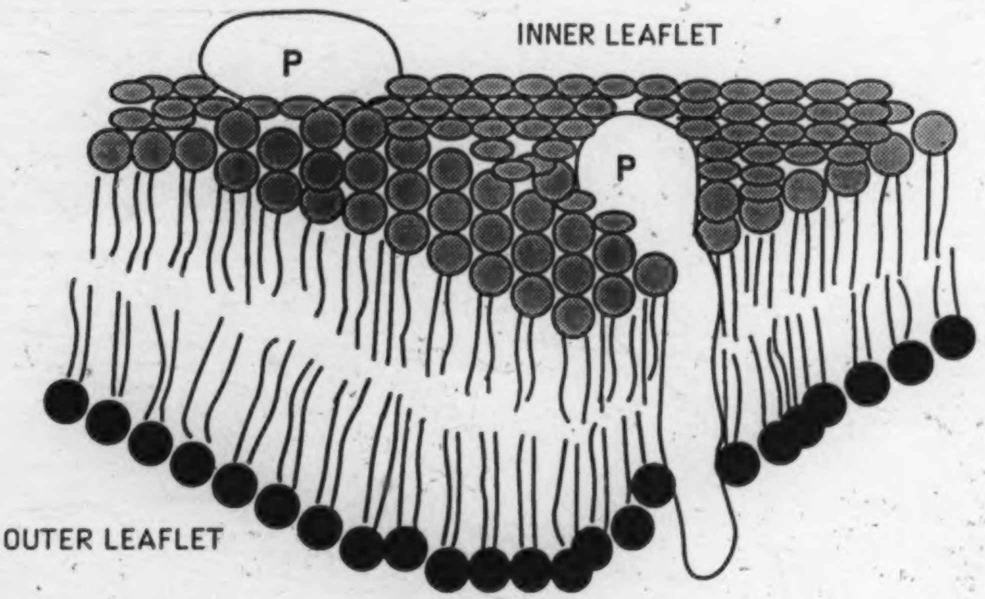
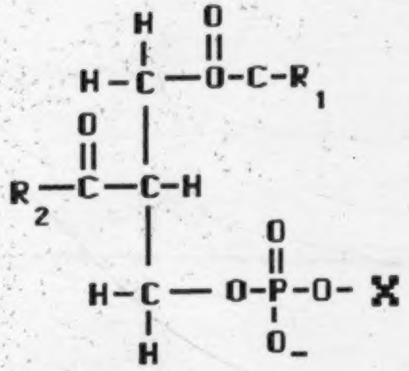

INNER LEAFLET
P
P
OUTER LEAFLET

FIGURE I.3.

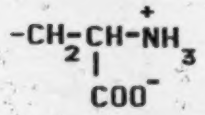
GENERAL STRUCTURE OF A GLYCEROPHOSPHOLIPID

This cartoon depicts the general structural features of a glycerophospholipid. To the glyceol-3-phosphate backbone two fatty acids, R_1 and R_2, are esterified. The head group molecule (**X**) is attached to the backbone via a phosphoester bond. The head groups and their respective phospholipids are: choline, phosphatidylcholine (PC); ethanolamine, phosphatidylethanolamine (PE); serine, phosphatidylserine (PS); glycerol, phosphatidylglycerol (PG); myo-inositol, phosphatidylinositol (PI); and phosphatidylglycerol, cardiolipin.



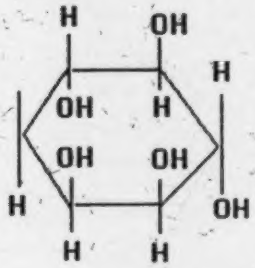
X = $-CH_2-CH_2-\overset{+}{N}(CH_2)_3$ = PC

$-CH_2-CH_2-\overset{+}{N}H_3$ = PE



$-CH_2-\underset{COO^-}{CH}-\overset{+}{N}H_3$ = PS

$-CH_2-CH(OH)CHOH$ = PG



= PI

AND WHEN X = PG = CARDIOLIPIN

FIGURE I.4.

de novo BIOSYNTHESIS OF PA, DG, AND CDP-DG

1) acylation of dihydroxyacetone-phosphate (DHAP) by the enzyme DHAP acyltransferase to form 1-acyl-DHAP; 2) reduction of acyl-DHAP to form 1-acyl-*sn*-glycerol-3-phosphate (lysoPA), catalyzed by the enzyme acyl-DHAP reductase; 3) the acylation of glycerol-3-phosphate (G-3-P) to lysoPA by the enzyme glycerol-3-phosphate acyltransferase; 4) lysoPA is then acylated by the enzyme 1-acylglycerol-3-P acyltransferase to form phosphatidic acid (PA); 5) hydrolysis of PA to form diacylglycerol (DG) by the enzyme phosphatidate phosphohydrolase (PAP); 6) PA may also be formed by the direct phosphorylation of DG, a reaction catalyzed by the ATP-dependent enzyme DG kinase; and 7) PA can react with a molecule of the nucleotide cytidine-5'-triphosphate (CTP) and form cytidine-5'-diphosphodiacylglycerol (CDP-DG). This reaction is catalyzed by the enzyme PA: cytidylyltransferase.

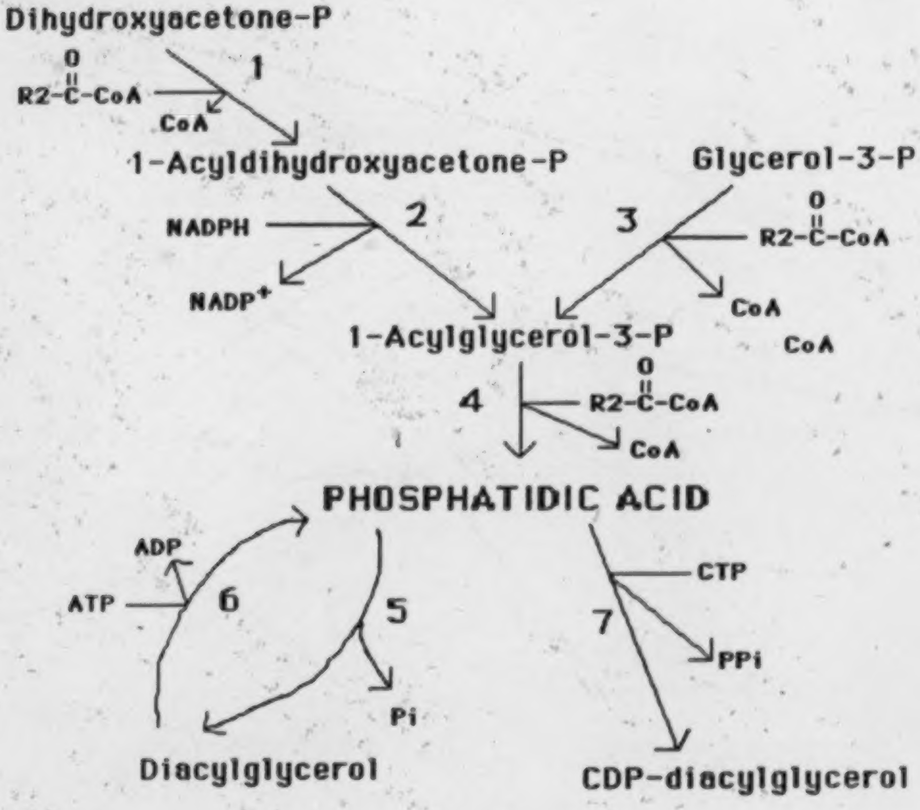

Dihydroxyacetone-P
R2-C(=O)-CoA
CoA
1
1-Acyldihydroxyacetone-P
Glycerol-3-P
NADPH
NADP+
2
3
R2-C(=O)-CoA
CoA
1-Acylglycerol-3-P
CoA
4
R2-C(=O)-CoA
CoA
PHOSPHATIDIC ACID
ADP
ATP
6
5
Pi
CTP
7
PPi
Diacylglycerol
CDP-diacylglycerol

FIGURE I.5.

MAJOR PATHWAYS OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS

1) The transfer of a phosphate group from a molecule of the nucleotide adeosine-5'-triphosphate (ATP) to choline to form phosphocholine, catalyzed by the enzyme choline kinase; 2) Condensation of phosphocholine with a molecule of the nucleotide cytidine-5'-triphosphate (CTP) to form cytidine-5'-diphosphocholine (CDP-choline), catalyzed by the enzyme CTP:Phosphocholine cytidylyltransferase; 3) The transfer of the phosphocholine moiety of CDP-choline to a diacylglycerol (DG) to form phosphatidylcholine (PC), catalyzed by the enzyme CDP-choline:1,2-diacylglycerol cholinephosphotransferase; 4) Reacylation of lysoPC by the enzyme acyl-CoA:lysoPC acyltransferase; 5) Reacylation of lysoPC by the enzyme lysoPC:lysoPC acyltransferase; and 6) Sequential transfer of methyl groups from three molecules of *S*-adenosylmethionine to one molecule of phosphatidylethanolamine to form one molecule of PC and three molecules of S-adenosylhomocysteine, catalyzed by the enzyme PE N-methyltransferase.

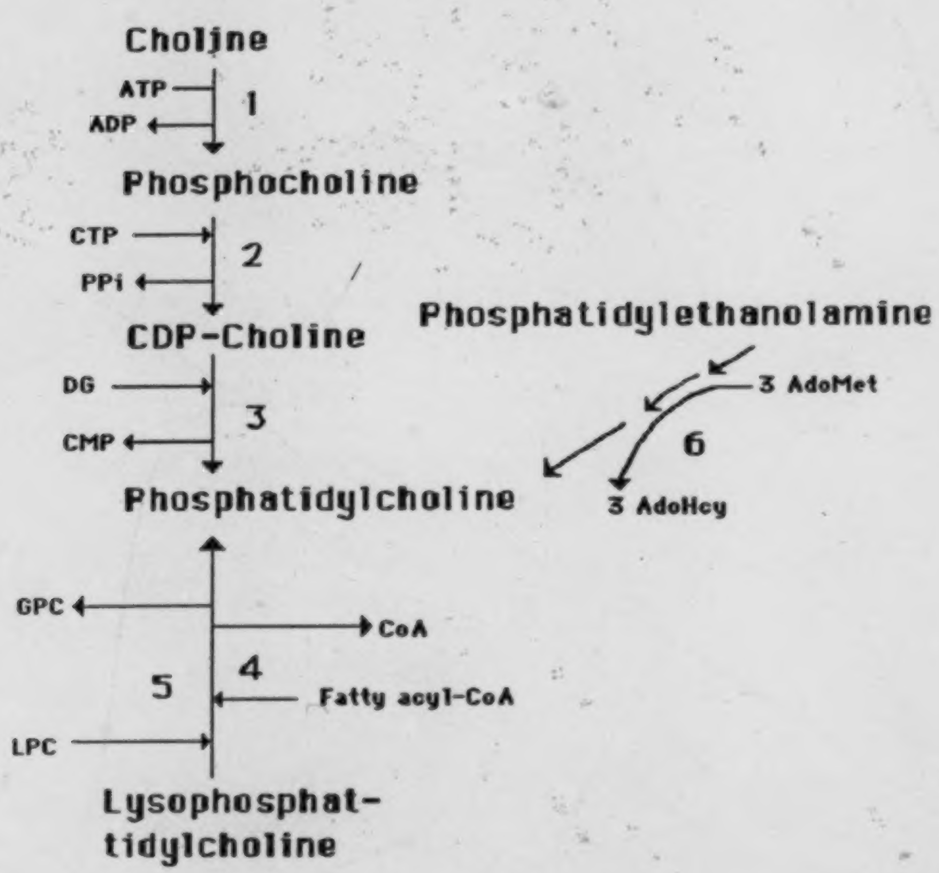

Choline
ATP
ADP
1
Phosphocholine
CTP
PPi
2
CDP-Choline
DG
CMP
3
Phosphatidylcholine
Phosphatidylethanolamine
3 AdoMet
6
3 AdoHcy
GPC
CoA
4
5
Fatty acyl-CoA
LPC
Lysophosphat-
tidylcholine

FIGURE I.6.

PATHWAYS OF PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS

1) The conversion of ethanolamine to phosphoethanolamine by the enzyme ethanolamine kinase; 2) Condensation of phosphoethanolamine with a molecule of the nucleotide cytidine-5'-triphosphate (CTP) to form cytidine-5'-diphosphoethanolamine (CDP-ethanolamine), catalyzed by the enzyme CTP:phosphoethanolamine cytidylyltransferase; 3) The transfer of the phosphoethanolamine moiety of CDP-ethanolamine to diacylglycerol (DG) to form phosphatidylethanolamine (PE), catalyzed by the enzyme CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase; 4) Reacylation of lysoPE with a fatty acyl-CoA, catalyzed by an acyltransferase; 5) Decarboxylation of phosphatidylserine in the mitochondria to form PE, catalyzed by the enzyme PS decarboxylase.

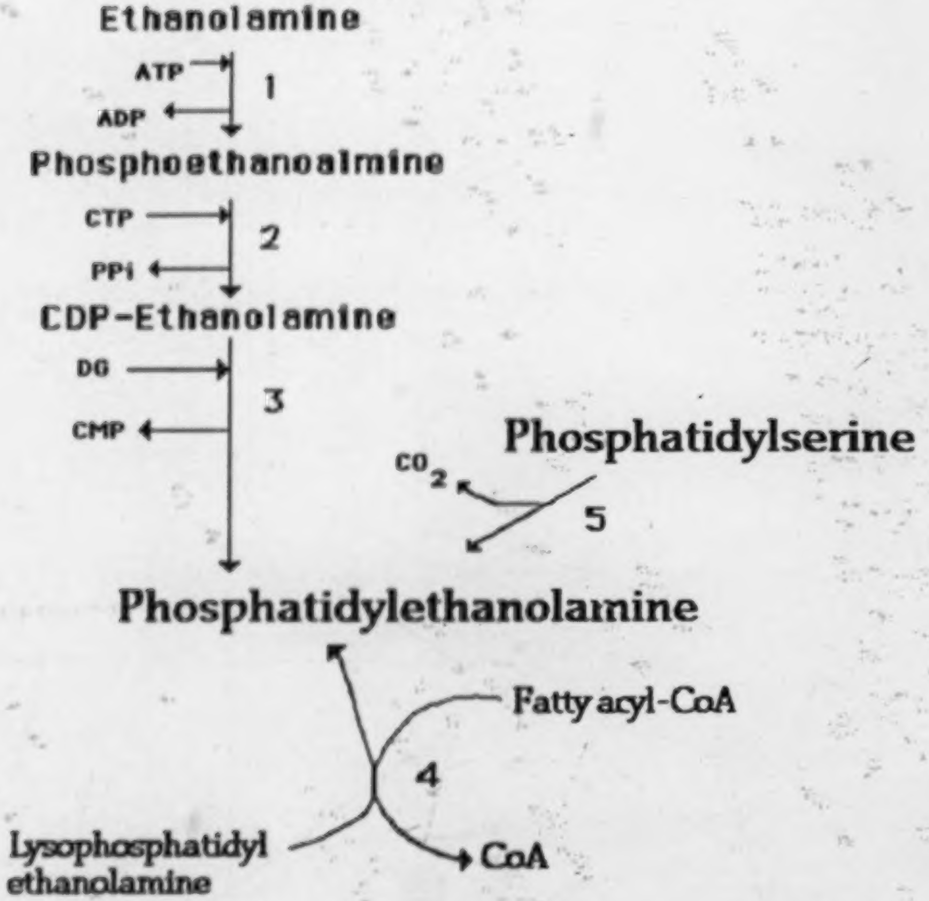
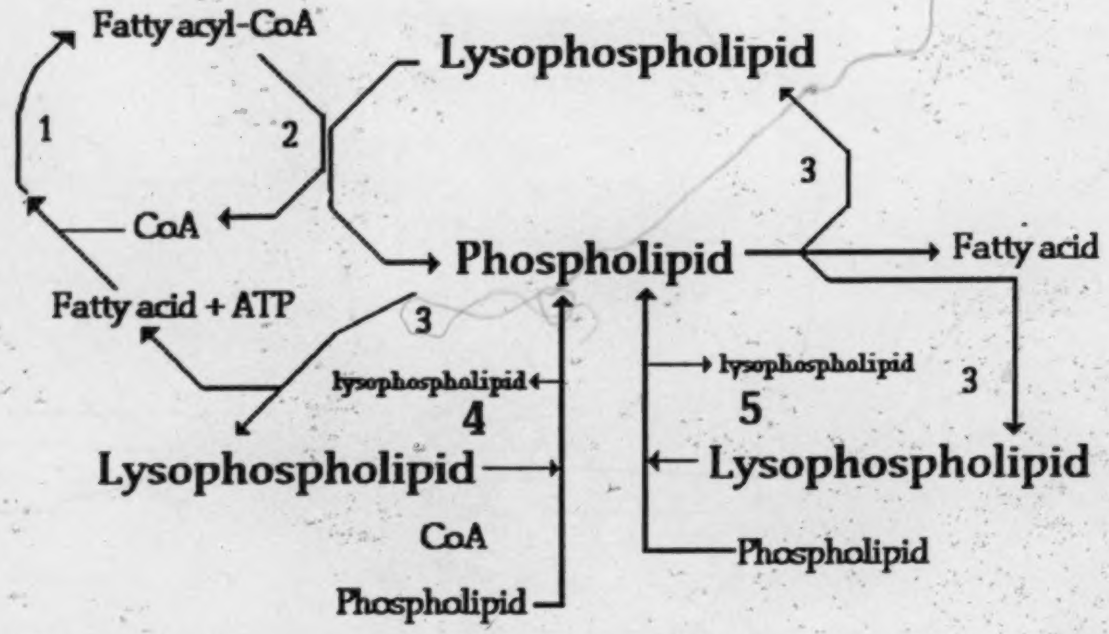

Ethanolamine
ATP
ADP
1
Phosphoethanoalmine
CTP
PPi
2
CDP-Ethanolamine
DG
CMP
3
Phosphatidylserine
CO2
5
Phosphatidylethanolamine
Fatty acyl-CoA
4
Lysophosphatidyl ethanolamine
CoA

FIGURE I.7.

POTENTIAL PATHWAYS FOR PHOSPHOLIPID REMODELING

1) Synthesis of fatty acyl-CoA from ATP and a fatty acid, catalysed by the enzyme acylCoA synthetase; 2) 'Lands pathway' reacylation of the lysophospholipid by transfer of an acyl group from an acyl-CoA molecule, catalysed by an acyltransferase; 3) phospholipase catalyzed removal of either the *sn*-1 or *sn*-2 fatty acyl group of a phospholipid molecule to form a lysophospholipid; 4) The putative 'CoA-dependent' reacylation of the lysophospholipid by transfer of an acyl group from a donor phospholipid; and 5) 'CoA-independent' reacylation of the lysophospholipid by transfer of an acyl group from a donor phospholipid.


Fatty acyl-CoA
Lysophospholipid
1
2
3
CoA
Phospholipid
Fatty acid
Fatty acid + ATP
3
lysophospholipid
4
lysophospholipid
5
3
Lysophospholipid
Lysophospholipid
CoA
Phospholipid
Phospholipid

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CHAPTER II.

Metabolism of [^{3}H]ethanolamine-labeled PE and PC[1]

INTRODUCTION

As discussed in chapter I, PC is made in rat liver via the CDP-choline pathway or by methylation of PE. Once PC is made, there is substantial evidence to suggest that the fatty acid composition is remodeled via deacylation and reacylation to yield PC molecular species enriched in the *sn*-2 position with polyunsaturated fatty acids, particularly arachidonic acid (Lands, 1965; Holub and Kuksis, 1978a). Such remodeling is also believed to occur with phosphatidylinositol (Holub and Kuksis, 1978b) and PE (Holub and Kuksis, 1978a; Åkesson et al., 1976; Åkesson et al., 1970a; Åkesson et al., 1970b; Trewhella and Collins, 1973; de Tomas, and Brenner, 1970; Åkesson, 1970; Holub and Kuksis, 1971). Somewhat inconsistent with the remodeling of PE at the *sn*-2 position is the finding that deacylation of PE in rat liver microsomes is largely due to a phospholipase A_1 specific for the *sn*-1 position (Waite, 1987; Holub, 1982). However, there are phospholipases A_2 in other organelles of rat liver (Waite, 1987) and, thus, remodeling of the fatty acid substituents is possible. Thus, it is generally believed that the molecular species composition of PE and PC is dictated by the specificities of the biosynthetic enzymes as well as by post-synthetic deacylation-reacylation.

We decided to re-evaluate the role of enzyme specificity and post biosynthetic remodeling of phospholipid acyl composition using primary hepatocytes maintained in culture. While extensive remodeling of PC molecular species was observed, there was no significant indication of remodeling of PE. Part of the PE was rapidly converted into PC which plateaued between 2 and 4 h and the remainder of PE further metabolized.

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MATERIALS AND METHODS

Materials

[1-^{3}H]ethanolamine, [*methyl*-^{3}H]choline and aqueous counting scintillant were from Amersham Corp., U.K. Choline, collagenase, ethanolamine, O-phosphoethanolamine and L-α-glycerolphosphorylethanolamine were from Sigma Chemical Co., St. Louis, MO. Dulbecco's modified Eagles medium (MEM, minus choline) was from Gibco, Grand Island, NY. Thin-layer chromatography silica gel 60 plates (0.2 mm) were obtained from Merck, Darmstadt, Germany. Culture dishes (60 mm) were from Becton, Dickinson & Co, Lincoln Park, NJ. Methanol and acetonitrile (HPLC grade) were obtained from Baker, Phillipsburg, PA.

Animals

Female Sprague-Dawley rats (125-175 gm) were maintained on standard Purina rat chow and water ad libitum.

Hepatocyte culturing

Rats were lightly anesthesized with diethylether prior to intraperitoneal injection of 5 mg pentobarbitone/100 g body weight. Hepatocytes were prepared by the collagenase perfusion technique (Davis et al., 1979) as previously described (Yao and Vance, 1988). Briefly, livers were perfused through the portal vein with 40-45 ml of Hanks' solution (Ca^{2+} and Mg^{2+} free) supplemented with 20 mM glucose, 25 mM Hepes, 0.5 mM EGTA and 10 µg/ml insulin. This was followed by 50-75 ml of collagenase (6 mg/ml) dissolved in Hanks' buffer. The liver was excised and further digested in 10 ml of collagenase solution by gentle agitation at 37°C. Approximately 30 ml of Dulbecco's MEM containing 17% fetal bovine serum was added and the cell suspension was filtered, centrifuged (50 x g for 2 min), washed three times with 40 ml MEM before plating. Cells were plated, in 3 ml of Dulbecco's MEM (plus 17% fetal bovine serum) containing 28 µM choline, at a density of 3 x 10^6 cells/dish and incubated for 12 to 18h in a 37°C incubator with a 5% CO_2 atmosphere.

Pulse chase experiments

1 h prior to experiments, the medium was replaced with serum-free MEM and the cells were equilibrated. Fifteen µCi of [1-^{3}H]ethanolamine or [*methyl*-^{3}H]choline was added to each dish and the cells were incubated for 1 h. The medium was replaced with serum-, choline- and ethanolamine-free Dulbecco's MEM containing either 100µM choline in the [^{3}H]choline study or 100 µM ethanolamine and choline in the [1-^{3}H]ethanolamine study. The cells were than incubated at 37^{o}C until the end of the chase.

Lipid extraction and isolation of PE and PC

At various times the cells were washed with ice-cold phosphate-buffered saline and harvested in 1.5 ml methanol/H_2O (1:1, v/v). The cells were scaped from the dish and after homogenization, by sonication, 1.2 ml was removed and placed in a 10 ml screw-capped tube. After the addition of 3.6 ml of chloroform (containing 0.05% butylated hydroxytoluene) and 50 µl of 0.1 N HCl the lipids were extracted (Folch et at., 1957). 2.0 ml of the aqueous phase was saved for analysis and the remainder was discarded. The organic phase was washed at least 2 times with 2.4 ml methanol/H_2O (1:1, v/v) and then dried under nitrogen. The lipid was dissolved in 1 ml of chloroform/methanol (2:1, v/v) and aliquots of this sample were used for further analysis. Phospholipid classes were separated by thin-layer chromatography (TLC) on silca Gel 60 using the solvent system of chloroform/methanol/acetic acid/water (50:30:5:2, v/v/v/v). Phospholipids were visualized by spraying with 0.2% dichlorofluorescein in 95% ethanol. In this solvent system PE, PS, PC, sphingomyelin, and LPC have R_f values of approximately 0.76, 0.54, 0.36, 0.21, and 0.16, respectively. This solvent system does not separate PC from lysoPE. However, this result did not affect this study as the mass of lysoPE and the amount of radioactivity associated with it was negligible in these experiments as determined by separating total lipids by 2-dimensional TLC. The first solvent system was chloroform/methanol/7 M ammoniumhydroxide (90:54:11, v/v/v) and the second solvent system was chloroform/methanol/acetic acid/water (45:20:6:1, v/v/v/v). PC and PE were extracted from the silica gel (Arvidson, 1968) and stored in chloroform/methanol (2:1, v/v) at -20^{o}C under nitrogen prior to HPLC analysis.

For analysis of aqueous ethanolamine catabolites the following was routinely done. To an aliquot of the upper phase (usually 100-250 µl) 0.4 µmole ethanolamine and 1 µmole each of ethanolamine-phosphate and glycerolphosphoethanolamine were added as carriers and the sample was dried under a stream of air. The sample was dissolved in 100 µl of methanol/H2O (1:1, v/v) and 50 µl was analyzed by thin-layer chromatography on silica gel 60. The plate was developed in methanol/0.6% NaCl/28% NH_4OH (10:10:1,v/v/v) and the bands corresponding to ethanolamine (E), ethanolamine-phosphate (E-P) and glycerolphosphoethanolamine (GPE) were scraped into scintillation vials and counted. E, E-P and GPE had R_f values of 0.23, 0.44 and 0.65, respectively.

HPLC analysis of PC and PE molecular species

Labeled PC and PE were separated on a 4.6 x 150 mm Pecosphere C18 reverse phase column (Perkin-Elmer Corp., Norwalk, CT) as previously described (Ridgway and Vance, 1988; Patton et al., 1982). Briefly, 250-350 nmole of PE or PC was dried down under nitrogen just prior to analysis. The phospholipid was dissolved in 30 µl of absolute ethanol and 25 µl was injected onto the HPLC column. The phospholipid was eluted using an isocratic system consisting of methanol/H_2O/acetonitrile (905:70:25, v/v/v) containing 20 mM choline chloride. The elution of molecular species was monitored at 205 nm and as such the solvent had to be purged with Helium gas. Individual peaks were collected and the percent distribution of radioactivity and lipid phosphorus (Rouser et al., 1986) determined. A typical elution profile for PC is shown in Fig. II.1. The major molecular species were identified by transmethylation of individual peaks with 14% BF_3 in absolute methanol (Pierce Chemical Co.). The resulting fatty acid methyl esters were analyzed by gas-liquid chromatography on a 6' x 1/8" column of 100-120 mesh Supelcoport (Supelco Inc., Bellefonte, PA) coated with 5% diethylene glycol succinate using temperature programming. This analysis indicated that the identity of the peaks shown in Fig. II.1 were correct and in agreement with previously published results (Ridgway and Vance, 1988; Patton et al., 1982)

RESULTS

Metabolism of [^{3}H-methyl]Choline-Labeled PC

Cultured rat hepatocytes were incubated for 1 h with [*methyl*-^{3}H]choline and chased for up to 24 h with 100 µM choline. As expected, the [^{3}H-*methyl*]choline was rapidly incorporated into PC at the end of a 1 h pulse period (Table II.1.; Fig. II.2). The molecular species labeled to the largest extent were 16:0-22:6 (I), 16:0-20:4 (II), 16:0-18:2 (III) [18:1-18:2 was poorly resolved from 16:0-18:2 (Ridgway and Vance, 1988) and were therefore collected in one fraction], 18:0-22:6 (IV), 18:0-20:4 (V), and 18:0-18:2 (VI) [18:0-22:5 was collected with 18:0-18:2]. During the chase period there was a change in the percent distribution of labeled molecular species so that after 12 h the radioactivity in PC reflected the mole percent distribution of PC (Table II.1.). The largest changes were seen in a decrease in the labeling of 16:0-22:6 and an increase in the percent labeling of 18:0-20:4. In contrast there was no apparent change in the percent distribution of label in 16:0-20:4-PC.

From measurements of the masses of the major molecular species, we were able to calculate the specific radioactivity of [*methyl*-^{3}H] choline-labeled PC (Fig. II.2.). In every case the specific radioactivity increased for up to 12 h. This result was expected since during the chase period there would be extensive synthesis of PC from the large pool of phospho-[*methyl*-^{3}H]choline in the hepatocytes (Pritchard and Vance, 1981). The data in Table II.1. and Fig. II.2. are also consistent with deacylation-reacylation of some molecular species (a decrease in I and II and an increase in V and VI). These transformations are not proven in this investigation but agree with earlier studies on remodeling of PC (Lands, 1965; Holub and Kuksis, 1978a).

Metabolism of [^{3}H]ethanolamine-labeled PE

In a similar manner to the choline experiments, hepatocytes were incubated with [^{3}H]ethanolamine for 1 h and chased for up to 24 h with excess unlabeled 100 µM ethanolamine. Fig. II.3 and Fig. II.4. show the incorporation of label into cellular PE and PC. There was extensive decay of radioactivity in PE during the 0 to 12 h chase period and a less significant decay of radioactivity in PE during the 12 to 24 h chase period. In the early hours of the chase the major metabolic fate of PE in liver cells is the conversion to PC by methylation with *S*-adenosylmethionine (Vance and Ridgway, 1988). Between 2 and 4 h of the chase the incorporation of [^{3}H]ethanolamine-labeled PE to PC reached a maximum and did not change significantly during the subsequent 20 h chase period (Figs. II.3. and II.4.). Apparently very little PE was methylated after 2 h of chase. This suggests the surprising conclusion that for the most part only newly-synthesized PE is used by the PE methyltransferase. Alternatively, the flattening of the curves for PE conversion into PC (Figs. II.3. and II.4.) might simply reflect a steady state where the synthesis of PC by methylation was balanced by metabolism of the PC. If this were the case, the radioactivity in PC should have declined between 12 and 24 h when very little labeled PE is being metabolized. This is clearly not the case as shown in Fig. II.3.

It is noteworthy in Fig. II.3. that the proportion of labeled PE converted to PC varied extensively among three experiments even though the incorporation into PE was very similar after the three experiments had been normalized. The reason for this observation and the need to normalize the data is that during the course of isolating the PE and PC from the experiments depicted in Fig. II.3. (which were used for HPLC analysis) we were more interested in the purity of fractions than in the recoveries. When these experiments were carefully repeated (Fig. II.4.) without HPLC analysis, we had no need to normalize the data and the variability previously seen was absent. To determine the nature of the PE catabolites we analysed the organic and aqueous components from both the cells and the medium of the experiments depicted in Fig. II.4. As shown in Table II.2. during the first 2 h of the chase approximately 85% of the label lost from PE is converted to PC. During the 6-12 h chase period all the label lost from PE was accounted for by an increase in cell and medium [^{3}H]ethanolamine-labeled GPE, ethanolamine-phosphate and ethanolamine (39, 33, and 24%, respectively). Approximately 68% of the radiolabel lost from PE was secreted into the medium, the major catabolite being GPE (Table II.2.). Radiolabel lost from the secretion of PE accounted for only 2%. Thus it would appear that PE, made by the CDP-ethanolamine pathway, is a preferred substrate for the PE-methyltransferase and that if not converted to PC it is rapidly degraded.

Remodeling of [^{3}H]Ethanolamine-Labeled PC but not [^{3}H]Ethanolamine-Labeled PE

The [^{3}H]ethanolamine-labeled PE and PC, isolated from the experiments depicted in Fig 3, were fractionated into molecular species by HPLC. The percent distribution of label in PE molecular species is shown in Table II.3. In agreement with earlier studies (Åkesson et al., 1976; Åkesson et al., 1970a; Trewhella and Collins, 1973; Åkesson, 1970; Holub and Kuksis, 1971) which described the major labeled molecular species as a hexaenoate-rich fraction, labeled 16:0-22:6-PE at the end of the pulse accounted for 44% of the incorporation of [^{3}H]ethanolamine into PE. During the 24 h chase period, the percent distribution in 16:0-22:6-PE decreased by 3-fold to 15%, closely approaching the mole % of this species in cellular PE (Table II.3.). Concomitantly, the percent incorporation of label into 18:0-20:4-PE increased over the 24 h chase period from 16 % to 44 %. The initial and incorrect conclusion from these data was that there had been a dramatic and complex set of deacylation-reacylation reactions that resulted in the conversion of 16:0-22:6-PE into 18:0-20:4-PE.

The correct interpretation of the pulse-chase experiment became apparent after the specific radioactivities of the major molecular species of PE were determined (Fig II.5.). Initially, the highest specific activity was observed in 16:0-22:6-PE (I) and this rapidly decayed (by 50% in the first 2 h). If this species were being converted into 18:0-20:4-PE (V), or any other species of PE, we should have observed an increase in the specific radioactivities in these molecular species with time. Instead, the specific activity did not increase in any of the molecular species of ethanolamine-labeled PE (Fig II.5.). The lack of an increase in total activity in 18:0-20:4-PE (V) (Fig. II.6), or any other species, might reflect a steady state where the synthesis of 18:0-20:4-PE (V) by remodeling of 16:0-22:6-PE is balanced by a rapid rate of catabolism. If this were the case we should have seen a dramatic increase in the specific activity of species V between 2 and 6 h as was observed for PC (see Figs. II.2. and II.7.). Moreover, from the data in Tables II.2. and II.3. it can be calculated that during the 6-12 h period of the chase there is a net loss of 1.12×10^6 cpm from species I, II and IV. During this same period there is a loss of 0.64×10^6 cpm from species V. If species I, II and IV were being remodeled into species V which was subsequently degraded, nearly 1.2×10^6 cpm would have to be lost from this species.

Thus, PE was not being remodeled to any significant extent, but rather the PE was being degraded by phospholipases to GPE and other catabolites.

The rates of decay of radioactivity in [^{3}H]ethanolamine-labeled molecular species of PE, from the experiment depicted in Fig II.3., were calculated and are shown in Fig II.6. For the total cellular PE and four (I, II, III, VI) of the six major molecular species, the rate of decay of radioactivity is clearly bimodal. A more rapid rate is observed in the initial 12 h followed by a slower rate of disappearance of radioactivity. The other two molecular species (IV, V) may also have both rapid and slow decay rates which are hard to distinguish. Analyses were also done on the data presented in Fig II.4 and Table II.3. with similar results (data not shown). From the data in Fig II.6. we can estimate the half-time for turnover of the molecular species and calculated the turnover time (T_t) (Ziversmit et al., 1948). The values are presented in Table II.4. In the course of our experiments we also measured the pool sizes (r) for PE and each major molecular species. Thus, we calculated the turnover rates (p) (Ziversmit et al., 1948) (Table II.4.). Species I (16:0-22:6-PE) had the fastest turnover time and turnover rate of all PE species. Species V (18:0-20:4-PE) had an initial turnover time almost twice that of any other molecular species and the turnover rate was 50% that of species I (16:0-22:6-PE). Since the turnover rate of 18:0-20:4-PE was in the same range as that of the other molecular species except 16:0-22:6-PE (Table II.4), the long turnover time simply reflects the large pool of 18:0-20:4-PE in the cells. In addition, from the decay of radioactivity between 12 and 24 h the slower turnover values [T_{t2} and p2] for the pools of PE have been calculated (Table II.4.).

As mentioned earlier, the major metabolic fate of PE during the early part of the chase is the conversion to PC. Thus, after the 1 h pulse period there was significant incorporation of [^{3}H]ethanolamine into PC (Table II.5.; Fig. II.7.; Fig. II.8; Fig. II.9.). The % distribution of the label in PC resembled the labeling of precursor PE at 0 h. As might be expected from the labeling pattern of PE (Table II.3.), the major species of PC labeled at the end of the pulse was 16:0-22:6-PC. The specific radioactivity of 16:0-22:6-PC (I) increased 2-fold during the first 2 h of the case and decreased by 3-fold during the subsequent 10 h of chase (Fig. II.7). The total incorporation of [^{3}H]ethanolamine into PC was very rapid in the first 2 to 4 h of the chase and did not change significantly during the subsequent 20 h chase period (Figs. II.3. and II.4.). However, during this same period there was extensive change in the total and percent distribution of label in PC species (Fig II.8., II.9. and Table II.5, respectively). A striking precursor-product relationship between 16:0-22:6-PC (I) and 18:0-20:4-PC (V) was observed (Fig II.8.). Taken together, it seems that there was significant remodeling of [^{3}H]ethanolamine-labeled PC in the hepatocytes.

DISCUSSION

Newly Synthesized PE is the Substrate for Methylation to PC

One surprising result from these studies was that after 2 h, further methylation of labeled PE was minimal even though there was large amounts of radioactivity in PE which could potentially be used for PC synthesis (Figs. II.3. and II.4. 2). Thus, newly made PE, rather than the bulk of cellular PE, is preferentially methylated to PC. Other studies suggest this may also be true for phosphatidylserine-derived PE (Vance, J. E., 1989).

One possibility would be that once the radioactive PE equilibriated with the unlabeled PE in the cell, the specific activity of PE would be too low to observe an increase in incorporation of label into PC. We have calculated that the specific activity of PE after 4 h of chase in the [^{3}H]ethanolamine-plulse-chase experiment would be 25,349 cpm/nmol PE assuming complete equilibriation. During the next hour the turnover rate (p) of PE would be 21.9 nmol/h (Table II.5.). Thus, during that hour period approximately 555,000 cpm would disappear from the PE. As is evident from Fig. II.4. there was no change in the amount of label incorporated into PC. A change of that magnitude should have been apparent during that and subsequent hours of chase. Fig. II.3. shows that [^{3}H]ethanolamine-labeled PC turnover is very slow. Thus, it is unlikely that there was an increase in labeled PC during the 4-5 h time period that was compensated by catabolism of PC. Moreover, as mentioned in the results section, radioactivity lost from PE can be accounted for by an increase in glycerolphosphoethanolamine, phosphoethanolamine and ethanolamine. Taken together there appears to be negligible conversion of PE to PC after the initial 4 hours of chase.

The bulk of PE synthesis from CDP-ethanolamine occurs on the endoplasmic reticulum and this is also the major location of the methyltransferase (Vance, J.E. and Vance, D.E., 1988). The implication is that the labeled PE flows from the site of the methyltransferase on the endoplasmic reticulum shortly after biosynthesis and does not return to this site in significant amounts. Once PE has left the vicinity of the PE methyltransferase, the PE is metabolized by phospholipases to GPE and other catabolites. The location of these phospholipases is unknown. Because these enzymes are found in virtually all subcellular compartments (Waite, 1987), it may be very difficult to identify the sites of PE catabolism. It is known that PE is delivered to other membranes at apparently different rates with preferential labeling of the plasma membrane and Golgi compared to the mitochondria (Vance, J. E., 1988a). The fate of the labeled PE in these organelles is not clearly described. It is known that exogenously added PE can be incorporated into the plasma membrane of CHO cells and this PE is preferentially delivered to nuclear membranes, mitochondria and Golgi (Sleight and Pagano, 1985). Whether the flow of endogenously made PE occurs from plasma membrane to these organelles is not known.

Remodeling of the Molecular Species of PC, but not PE

Several technical advances in the past decade have made it possible to evaluate the remodeling of phospholipids in liver cells, a phenomenon which was described by Lands nearly 25 years ago (Lands, 1965). Particularly important have been the ability to culture hepatocytes (Davis et al., 1979) and the development of HPLC methods for resolving molecular species of PE and PC (Patton et al., 1982). The present experiments show that extensive remodeling of PC does occur in cultured hepatocytes. The lack of remodeling of PE was surprising particularly since there were striking changes in the percent composition of 16:0-22:6-PE (I) (44 to 15%) and 18:0-20:4-PE (16 to 44 %) over a 24 h period. On first analysis, it seemed that the 16:0 species of PE were being converted to the 18:0 species. However, the specific radioactivity of the 16:0 species decreased more than 5-fold without an increase in the specific radioactivity of the 18:0 species (Fig. II.5.). Thus, a precursor-product relationship between these two molecular species was not evident. There was not significant remodeling of any of the PE species since in no instance was there an increase in the specific radioactivity of any of the species of PE (Fig. II.5.).

If phosphatidylserine were a major source of PE, it might have diluted newly synthesized PE such that a significant increase of specific radioactivity may not have been observed. However, an increase in the total radioactivity of the PE molecular species should have been detected. It is clear from the data in Fig. II.6. that there was not an increase in the total radioactivity associated with the six major molecular species of PE during 24 h.

Another surprise was the rapid decay of radioactivity in PE (Figs. II.3. and II.4). We have identified the major catabolic products of PE during the chase period (Table II.2). While the majority of PE could be degraded by phospholipase activities in various organelles (Waite, 1987), it may be metabolized by other routes. PE is thought to be a precursor for the synthesis of phosphatidylserine via a base exchange reaction (Kanfer , 1989). PE is also utilized for lipoprotein secretion although the PE derived from CDP-ethanolamine is preferentially not used (Vance, J. E. and Vance, D. E., 1986, Vance, J. E., 1988b). Recently, [^{3}H]ethanolamine was shown to be incorporated specifically into the protein elongation factor 1α of a human erythroleukemia cell line (Rosenberry et al., 1989). PE is a possible candidate for the donor of this ethanolamine moiety. An enigma is why PE, made by the CDP-ethanolamine pathway, should be degraded so quickly?

In Table II.4. it is apparent that the turnover rate (p) of 16:0-22:6-PE is 2-3 times faster than the turnover rate of the five other major molecular species of PE. Consistent with these observations are the findings of Kuksis et al. (1975) who have shown that 16:0-containing species turnover at a faster rate than do 18:0-containing species and that 22:6-containing species turnover at a faster rate than 20:4 containing species. This preferential degradation of 16:0-22:6-PE (I) (Table II.4. and Kuksis et al., 1975) probably reflects the *in vivo* specificities of the phospholipases and lysophospholipases involved in PE degradation. While Pind et al. (1985) have shown, using endogenously labeled rat liver microsomes, that *in vitro* 16:0-containing-PE species are preferentially degraded by phospholipase A_1, Holub (1982) has been unable to demonstrate any *in vitro* selectivity of phospholipase A_1 using exogenously labeled rat liver microsomes. A better understanding of the *in vivo* specificities of phospholipases is required.

There is good agreement between our studies and earlier publications showing that the hexaenoate fraction is the major initial biosynthetic product of PE (Åkesson et al., 1976; Åkesson et al., 1970a; Trewhella and Collins, 1973; Holub and Kuksis, 1971; Kanoh, 1969). Our data also agree with the preference of the ethanolaminephosphotransferase for 1-saturated-2-docosahexaenoyl-glycerol precursors for PE studied with rat liver microsomes (Holub, 1977). However, the lack of remodeling of PE in hepatocytes is contrary to the view that has prevailed over the past two decades (Holub and Kuksis, 1978a; Åkesson et al., 1976; Åkesson et al., 1970a; Åkesson et al., 1970b; Trewhella and Collins, 1973; de Tomas, and Brenner, 1970; Holub and Kuksis, 1971). Examination of this earlier literature showed that the evidence for remodeling of PE molecular species was not conclusive. The following differences in experimental design may explain the apparent discrepancy between ours and previous conclusions.

(a) Earlier protocols involved the injection of [^{3}H]glycerol or [^{3}H]fatty acid into intact animals, isolation of the phospholipids up to 60 min later and fractionation of molecular species by argentation chromatography (Åkesson et al., 1970a; Åkesson et al., 1970b; Åkesson, 1970; Holub and Kuksis, 1971). Argentation chromatography does not allow for complete separation of molecular species. Thus 16:0-22:6-PE would be isolated with 18:0-22:6-PE. Even when used in association with gas chromatography (Holub and Kuksis, 1971) or counter current analysis (Trewhella and Collins, 1973), this method is crude when compared to current separation methods based on high pressure liquid chromatography (Patton et al., 1982). In addition [^{3}H]glycerol, [^{3}H]fatty acid or [^{32}P]phosphate would be incorporated into PE via decarboxylation of phosphatidylserine as well as via the CDP-ethanolamine pathway. It is possible, that PE made by decarboxylation of phosphatidylserine could be modified significantly by deacylation-reacylation even though ethanolamine-derived PE is not remodeled.

(b) Previous studies were largely done in whole animals and it is possible that isolated hepatocytes might behave differently. We consider this unlikely since isolated hepatocytes retain most functions of liver cells in whole animals (Pelech and Vance, 1984; Davis et al., 1979; Yao and Vance, 1988).

(c) Another potential source for misinterpretation of previous data might have been that the time courses were generally less than 60 min and it now seems that this may not be sufficient time to evaluate PE metabolism.

(d) Part of the problem may have been expression of some data as percent. As clearly demonstrated in the present study, such tabulation of data might lead to erroneous conclusions.

(e) Finally, the idea of remodeling of PE was undoubtedly influenced by the demonstration that injection of 1-acyl-*sn*-glycerophosphoethanolamine led to rapid labeling of the tetraenoic fractions (Åkesson et al., 1976; de Tomas and Collins, 1973). All of these factors taken together plus the results for remodeling of PC apparently made remodeling of PE seem plausible.

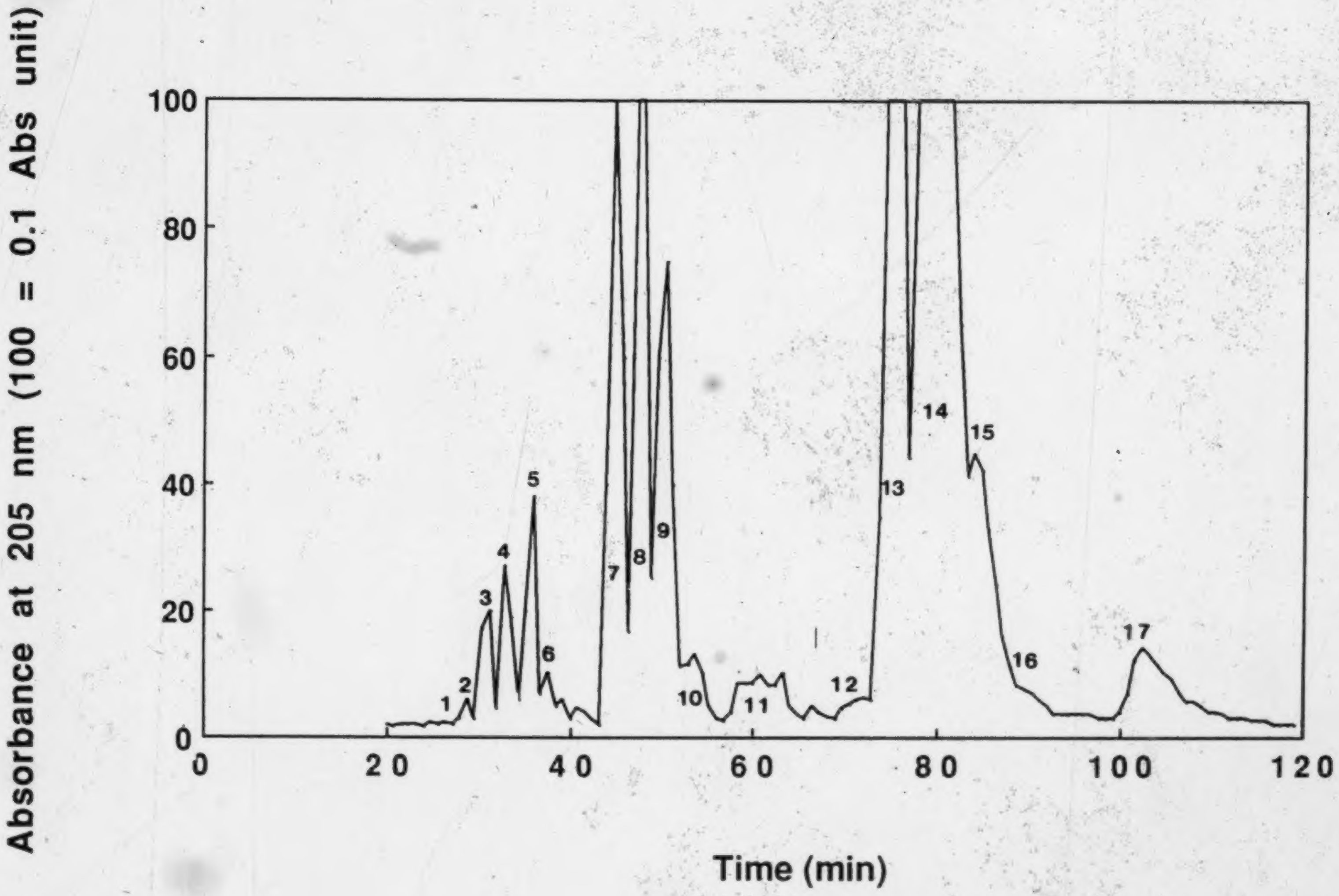
In contrast to PE metabolism, there does seem to be extensive remodeling of PC derived from either PE methylation or the CDP-choline pathway. This is indicated by a decreased specific radioactivity of 16:0-22:6-PC and an increased specific radioactivity of the major molecular species derived from CDP-choline and PE methylation (except for 16:0-22:6-PC) and the extensive change in the percent distribution of labeled molecular species (Tables II.1. and II.5). For most of the molecular species the remodeling is achieved in 6 to 12 h. The increase in specific radioactivity of PC molecular species derived from CDP-choline pathway also reflects synthesis of PC from the large pool of phospho[^{3}H]choline during the chase period. A major objective of the remodeling reaction appears to be the generation of 18:0-20:4-PC.

Finally, there were significant differences in the turnover rates of 16:0-22:6-PE compared to 18:0-22:6-PE (Fig II.6 and Table II.4). Thus, simply having 18:0 fatty acid at the *sn*-1 position of PE instead of 16:0 decreased the turnover rate from 5.8 to 2.0 nmole/hr (Table IV). The reason for and the metabolic significance of this difference in turnover rates is mysterious.

FIGURE II.1.

TYPICAL HPLC ELUTION PROFILE OF RAT LIVER MICROSOMAL PC

Identity of the peaks are as follows: 1 & 2, 18:2-18:3, 14:0-22:6; 3 & 4, 16:1-18:2, 18:2-22:6; 5 & 6 18:2-20:4, 18:2-18:2; 7 (I), 16:0-22:6, 8 (II), 16:0-20:4; 9 (III), 16:0-18:2; 10 (III), 18:1-18:2; 11, 18:0-20:5, 16:0-22:5; 12, 16:0-18:1; 13 (IV), 18:0-22:6; 14 (V), 18:0-20:4; 15 (VI), 18:0-18:2; 16 (VI), 18:0-22:5 (n-3); 17, 18:0-22:5 (n-6). Roman numerals refer to molecular species described in this chapter.



Absorbance at 205 nm (100 = 0.1 Abs unit)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

FIGURE II.2.

SPECIFIC RADIOACTIVITY OF MOLECULAR SPECIES OF CELLULAR PC DERIVED FROM [methyl-^{3}H]CHOLINE

Molecular species of PC, isolated from the experiments depicted in Table II.1., were analyzed for radioactivity and phosphorus content as described under Materials and Methods. Values are the mean of four separate experiments ± S.E.M. The number on the x axis refer to the molecular species (I, 16:0-22:6, II, 16:0-20:4; III, 16:0-18:2 & 18:1-18:2; IV, 18:0-22:6; V, 18:0-20:4; VI, 18:0-18:2 & 18:0-22:5). The time of the chase period is indicated in the Figure.

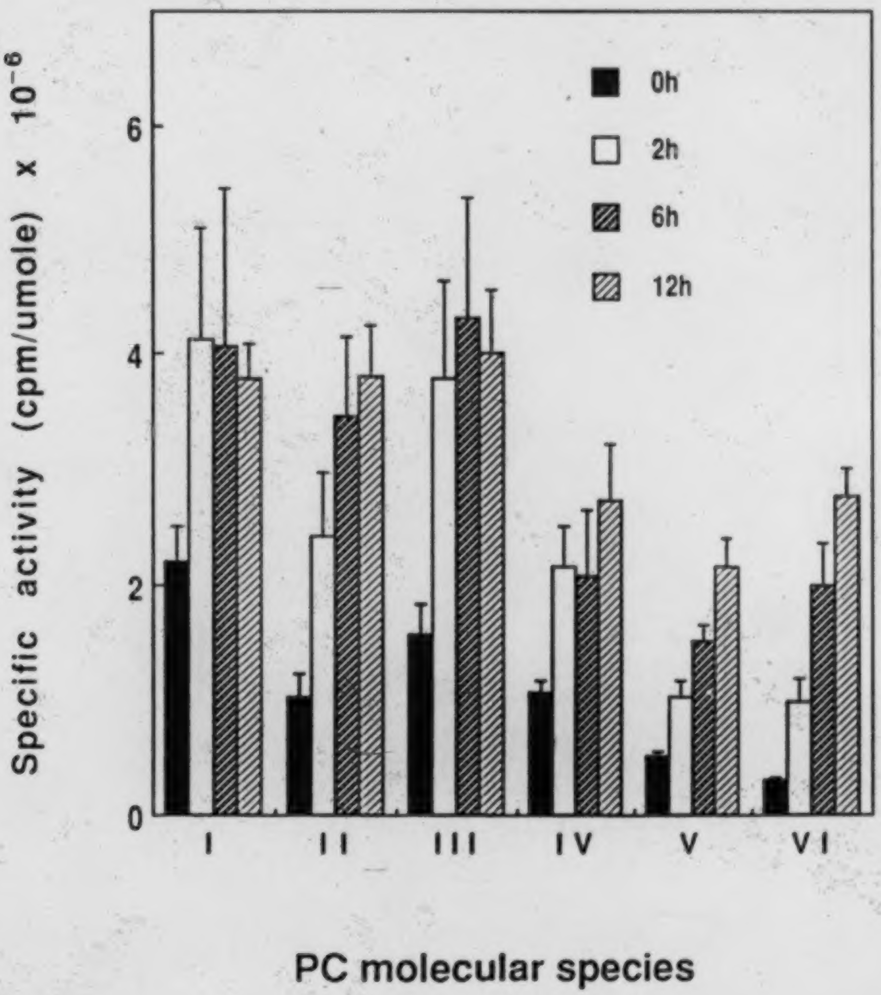

Specific activity (cpm/umole) x 10^{-6}
0
2
4
6
0h
2h
6h
12h
I
II
III
IV
V
VI
PC molecular species

FIGURE II.3.

TOTAL RADIOACTIVITY OF CELLULAR PE AND PC DERIVED FROM [^{3}H]ETHANOLAMINE (HPLC PREPARATION)

Total PE and PC, destined for HPLC analysis (see page 77), were isolated from three sets of experiments and analyzed for radioactivity as described under Material and Methods. Due to variations in the incorporation of labeled ethanolamine and the recoveries of PC and PE from experiment to experiment, it was necessary to normalize the data. The PE 0 h value for experiment #1 was arbitrarily chosen as the standard. To obtain the values for experiment #2 and #3 the data were multiplied by 2.125 and 0.536, respectively. The values are the mean ± SD for three experiments after normalization.

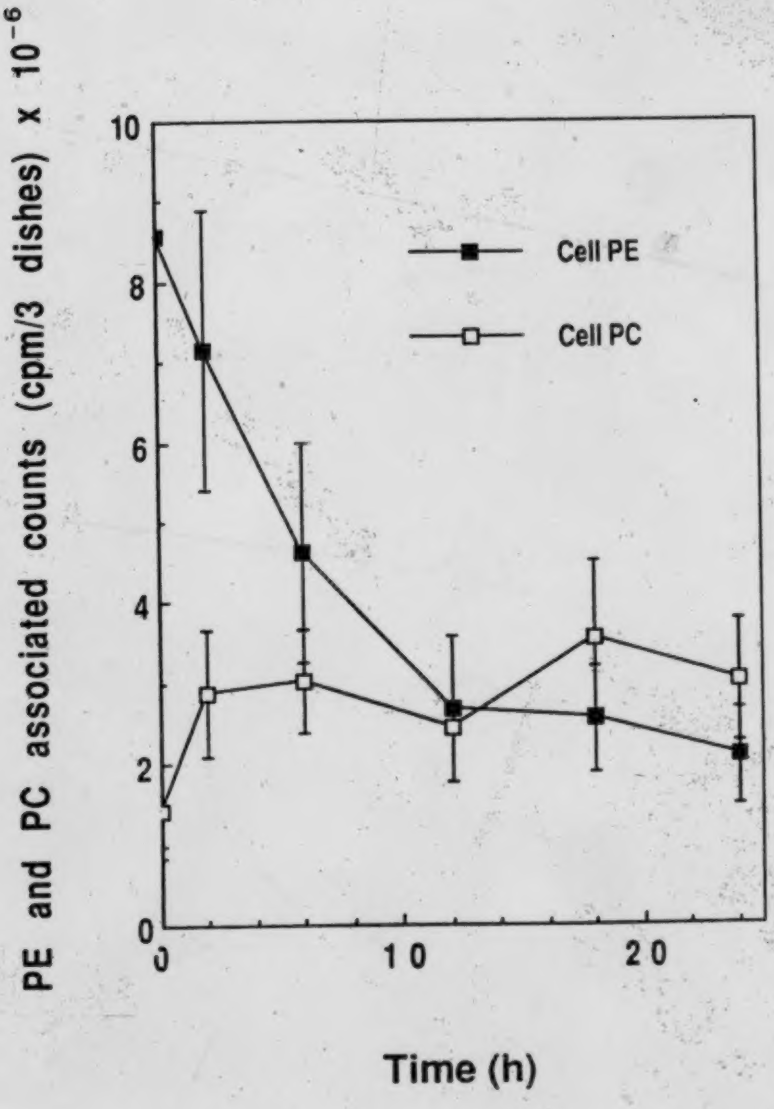

PE and PC associated counts (cpm/3 dishes) x 10^{-6}
10
8
6
4
2
0
0
10
20
Time (h)
Cell PE
Cell PC

FIGURE II.4.

TOTAL RADIOACTIVITY OF CELLULAR PE AND PC DERIVED FROM [^{3}H]ETHANOLAMINE (ANALYTICAL PREPARATION)

Total PE and PC, not destined for HPLC analysis, were isolated from three sets of experiments and analyzed for radioactivity as described under Material and Methods. This method allowed for better recoveries of label (greater than 95%), and the data did not need to be normalized. The values are the mean ± SD for three experiments.

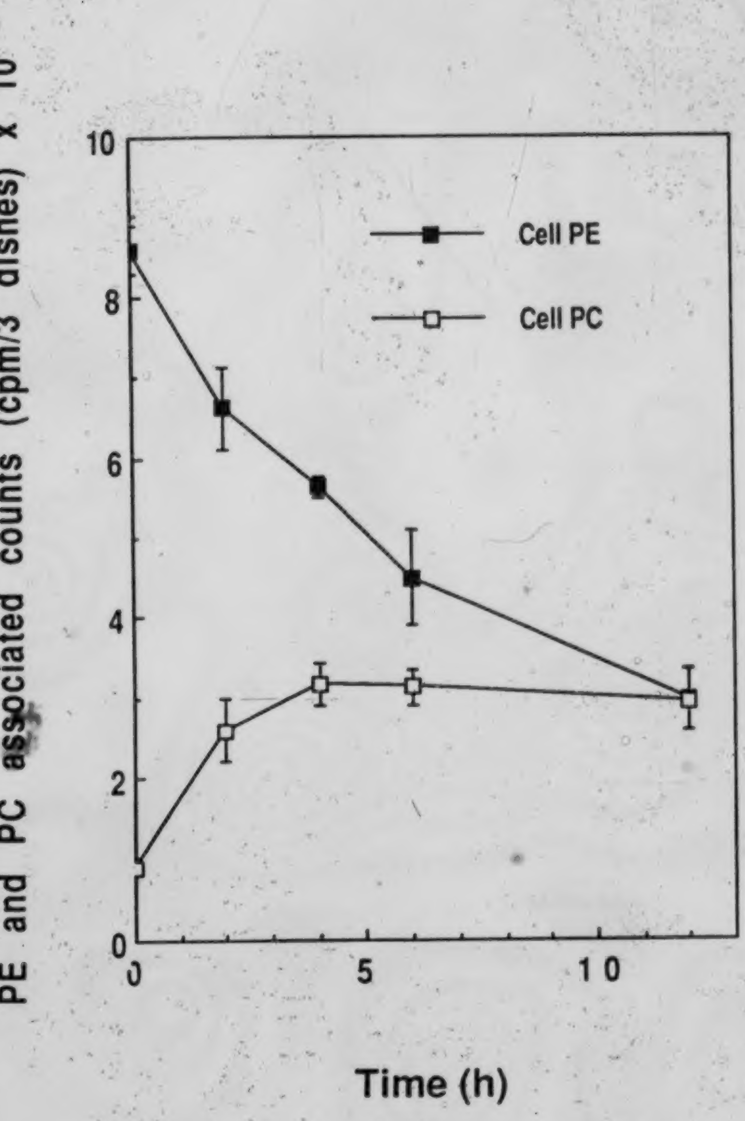

PE and PC associated counts (cpm/3 dishes) x 10^{-6}
10
8
6
4
2
0
0
5
10
Time (h)
Cell PE
Cell PC

FIGURE II.5

SPECIFIC RADIOACTIVITY OF MOLECULAR SPECIES OF CELLULAR PE DERIVED FROM [^{3}H]ETHANOLAMINE

Molecular species of PE, isolated from the experiments depicted in Table III.3., were analyzed for radioactivity and phosphorus content as described under Materials and Methods. Values are the mean of three spearate experiments ± S.E.M. The number on the x axis refer to the molecular species (I, 16:0-22:6; II, 16:0-20:4: III, 16:0-18:2 & 18:1-18:2, IV, 18:0-22:6; V, 18:0-20:4; VI 18:0-18:2 & 18:0-22:5).

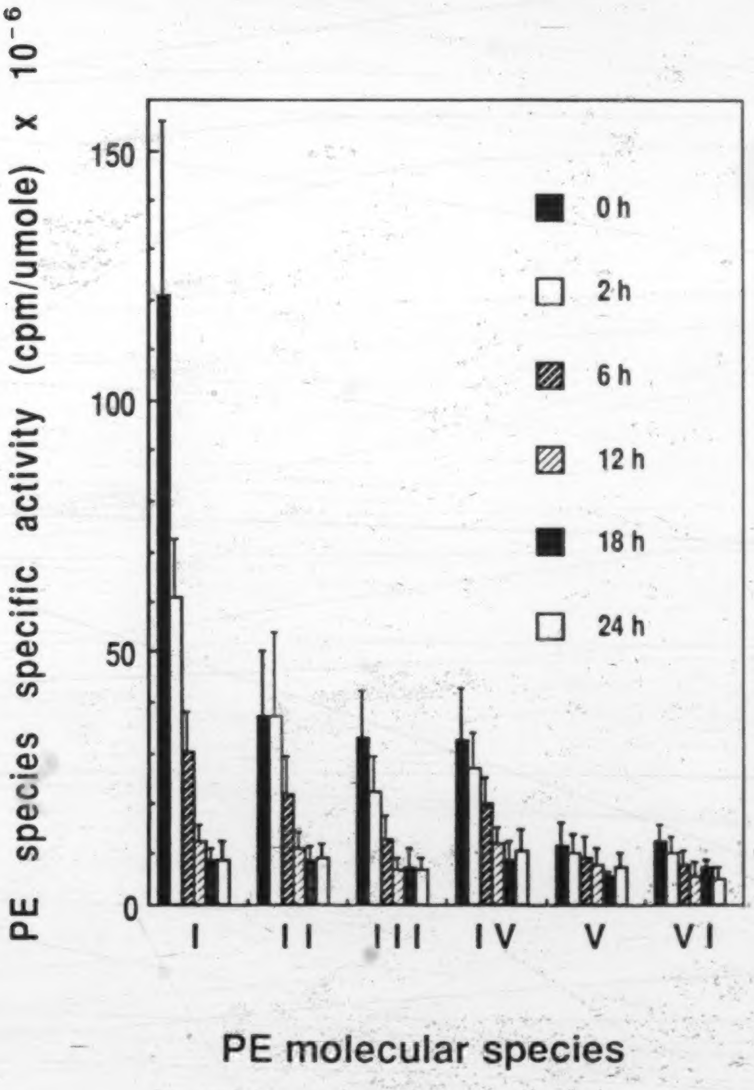

PE species specific activity (cpm/umole) x 10^{-6}
0
50
100
150
0 h
2 h
6 h
12 h
18 h
24 h
I
II
III
IV
V
VI
PE molecular species

FIGURE II.6.

DECAY OF RADIOACTIVITY FROM [^{3}H]ETHANOLAMINE-LABELED PE AND PE MOLECULAR SPECIES

Values were calculated by multiplying the percent distribution of individual molecular species (Table III.3.) by the average total PE radioactivity from three separate experiments (Fig. 4.). The identities of the molecular species are: (I, 16:0-22:6; II, 16:0-20:4; III, 16:0-18:2 & 18:1-18:2; IV, 18:0-22:6; V, 18:0-20:4; VI 18:0-18:2 & 18:0-22:5).

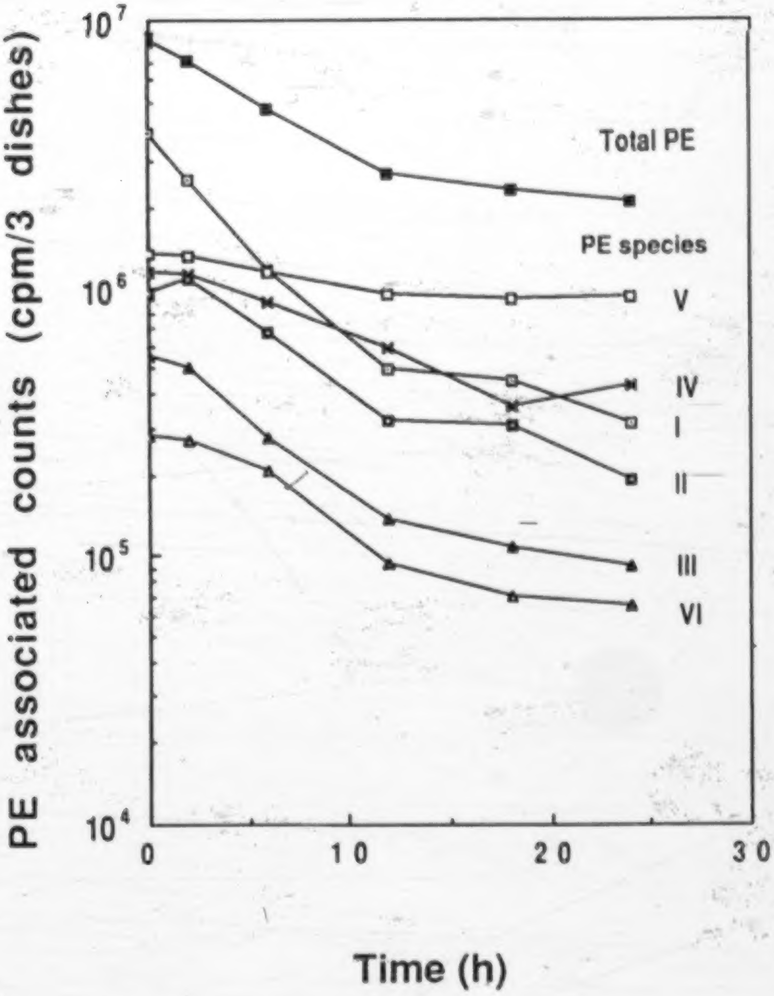

PE associated counts (cpm/3 dishes)
10^7
10^6
10^5
10^4
0
10
20
30
Time (h)
Total PE
PE species
V
IV
I
II
III
VI

FIGURE II.7.

SPECIFIC RADIOACTIVITY OF MOLECULAR SPECIES OF CELLULAR PC DERIVED FROM [^{3}H]ETHANOLAMINE

Molecular species of PC, isolated from the experiments depicted in Table III.3., were analyzed for radioactivity and phosphorus content as described under Materials and Methods. Values are the mean of three spearate experiments ± S.E.M. The number on the x axis refer to the molecular species (I, 16:0-22:6; II, 16:0-20:4; III, 16:0-18:2 & 18:1-18:2; IV, 18:0-22:6; V, 18:0-20:4; VI 18:0-18:2 & 18:0-22:5).

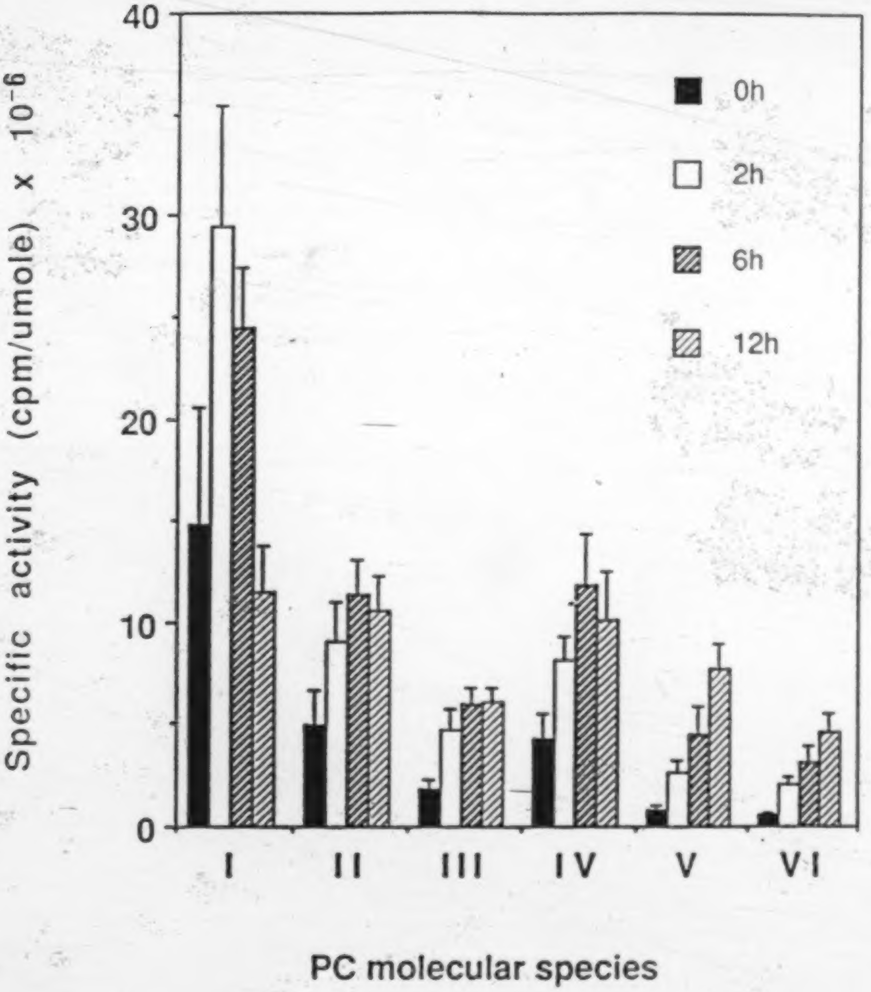

40
30
20
10
0
Specific activity (cpm/umole) x 10^{-6}
0h
2h
6h
12h
I
II
III
IV
V
VI
PC molecular species

FIGURE II.8.

REMODELING OF [^{3}H]ETHANOLAMINE-LABELED PC MOLECULAR SPECIES (16:0-22:6; 18:0-20:4)

Values were calculated by multiplying the percent distribution of individual molecular species (Table III.5.) by the average total PC radioactivity from three separate experiments (Table III.2.). This figure shows the precursor-product relationship between 16:0-22:6-PC (I) and 18:0-20:4-PC (V).

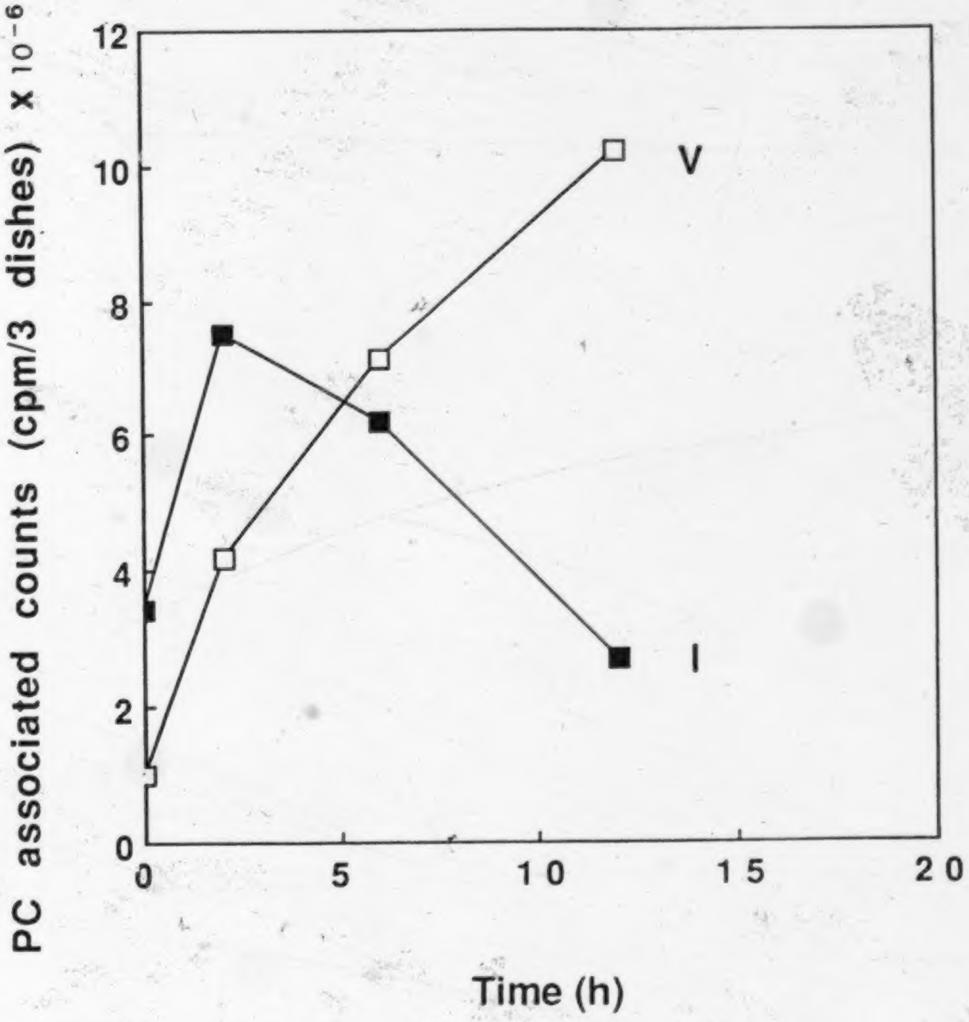

PC associated counts (cpm/3 dishes) x 10⁻⁶
0
2
4
6
8
10
12
0
5
10
15
20
Time (h)
V
I

FIGURE II.9.

REMODELING OF [^{3}H]ETHANOLAMINE-LABELED PC MOLECULAR SPECIES (16:0-20:4; 16:0-18:2 and 18:1-18:2; 18:0-22:6; 18:0-18:2 and 18:0-22:5)

Values were calculated by multiplying the percent distribution of individual molecular species (Table III.5.) by the average total PC radioactivity from three separate experiments (Table III.2.). This figure shows the metabolism of the other four major PC species (II, 16:0-20:4; III, 16:0-18:2 & 18:1-18:2; IV, 18:0-22:6; VI 18:0-18:2 & 18:0-22:5).

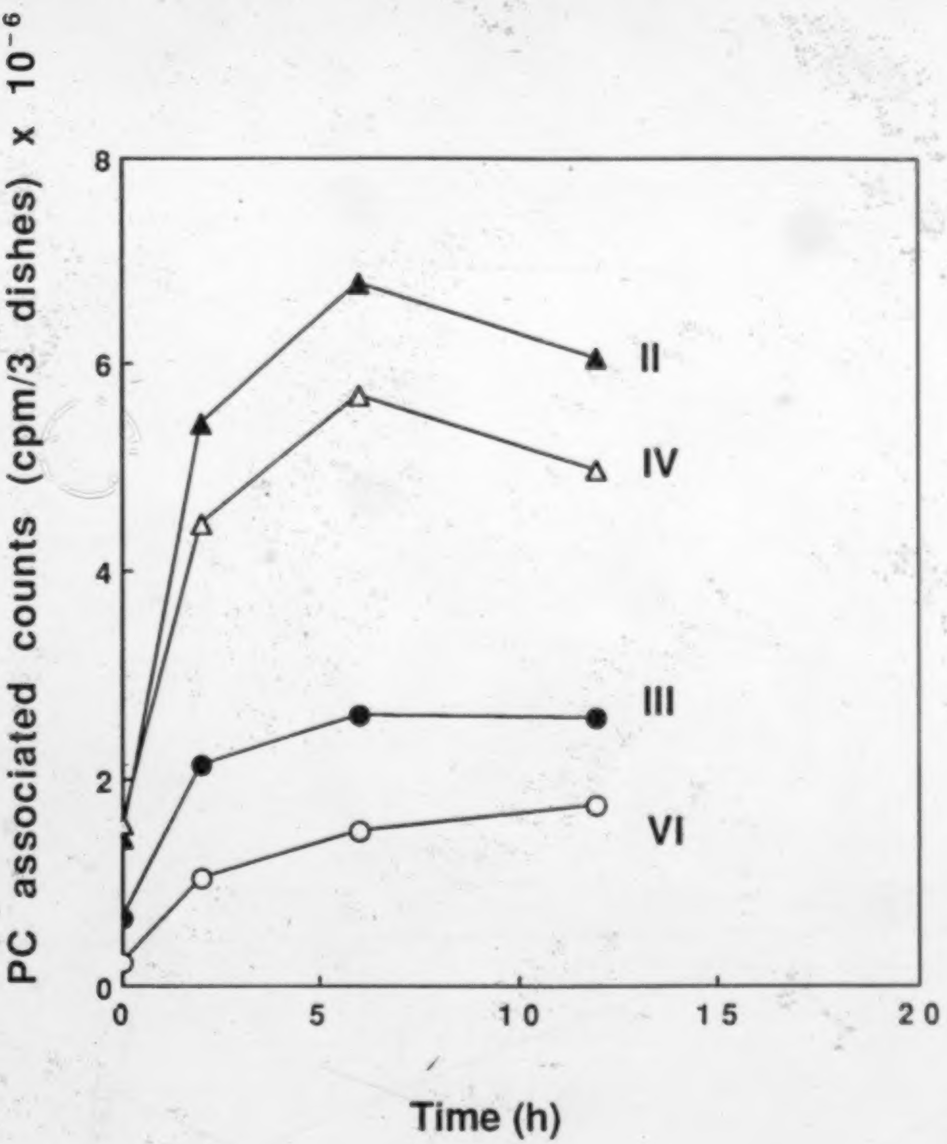

PC associated counts (cpm/3 dishes) x 10^{-6}
8
6
4
2
0
0
5
10
15
20
Time (h)
II
IV
III
VI

TABLE II.1.

PERCENT DISTRIBUTION OF [^{3}H]CHOLINE-LABELED PC MOLECULAR SPECIES IN HEPATOCYTES AS A FUNCTION OF TIME

Hepatocytes were prepared as described in the Materials and Methods. At the start of the experiment medium was replaced with fresh MEM (choline-deficient) and incubated for 30 min. The medium was then replaced with 2 ml choline-deficient MEM containing 7.5 uCi/ml[^{3}H]methyl choline and incubated for 1 h. At the end of the pulse the medium was replaced with MEM containing 100 µM choline. At the indicated times PC was isolated from 3 dishes of cells and molecular species analyzed as described in Materials and Methods. The results are expressed as the mean of 4 separate pulse-chase experiments ± S.D. The sum of the percentages in each experiment was 100%, but this was not the case when the average ± S.D. was summed.

Molecular Species	Time (hours)						mol% cellular PC
	0	2	6	1 2	1 8	2 4	
18:2-18:3,14:0-22:6	1.3 ±0.9	0.7 ±0.7	0.5 ±0.2	0.7 ±0.5	0.4 ±0.1	0.6 ±0.4	1.6 ±0.2
16:1-18:2,18:2-22:6	2.4 ±1.3	0.4 ±0.2	1.0 ±0.2	0.9 ±0.2	0.8 ±0.2	1.3 ±0.8	1.8 ±1.1
18:2-20:4,18:2-18:2	7 ±0.9	1.9 ±0.8	1.1 ±0.3	0.8 ±0.3	0.9 ±0.3	1.6 ±1.3	2.5 ±0.1
16:0-22:6	12.6 ±2.3	10.1 ±2.7	7.3 ±2.7	6.7 ±2.5	5.1 ±2.2	5.5 ±2.6	6.3 ±1.1
16:0-20:4	8.6 ±6.9	18.2 ±2.2	19.5 ±1.6	18.7 ±0.9	17.8 ±1.4	17.1 ±5.2	13.2 ±0.9
16:0-18:2,18:1-18:2	6.3 ±5.1	20.8 ±4.3	15.2 ±2.0	13.3 ±0.6	10.8 ±0.7	11.1 ±1.6	10.4 ±1.5
18:0-20:5,16:0-22:5	2.4 ±0.6	2.1 ±0.6	1.9 ±0.7	1.8 ±0.5	2.0 ±0.6	2.4 ±1.2	2.9 ±0.4
16:0-18:1	5.0 ±2.5	4.9 ±3.3	5.0 ±3.3	4.1 ±1.9	4.7 ±0.9	4.1 ±2.9	3.3 ±1.0
18:0-22:6	11.9 ±4.4	12.1 ±5.5	12.3 ±7.1	10.7 ±5.6	8.9 ±3.3	9.9 ±4.1	11.9 ±0.9
18:0-20:4	22.9 ±5.8	22.7 ±7.5	29.0 ±9.9	33.2 ±7.0	38.7 ±5.6	36.3 ±5.3	31.3 ±1.9
18:0-18:2,18:0-22:5	5.8 ±2.5	5.5 ±0.6	7.9 ±0.9	9.1 ±1.7	10.0 ±0.8	12.3 ±4.6	10.3 ±1.8
18:0-20:3,18:0-22.5	1.1 ±1.1	0.8 ±0.4	0.8 ±0.3	0.9 ±0.2	1.0 ±0.1	0.9 ±0.4	2.4 ±0.1

TABLE II.2.

CATABOLISM OF [^{3}H]ETHANOLAMINE-LABELED PE DURING A 12-H CHASE

Primary hepatocytes were cultured for 12-18 h in Dulbecco's MEM containing 17% fetal bovine serum. At the start of the experiment medium was replaced with fresh MEM without serum and incubated for 30 min. The medium was replaced with 2 ml MEM containing 7.5 uCi/ml [1-^{3}H]ethanolamine and incubated for 1 h. At the end of the pulse the medium was replaced with MEM containing 100 µM ethanolamine. At the indicated times the major PE catabolites were isolated from the medium and cells as described in the Methods. Results are expressed as the mean of 3 separate pulse-chase experiments ± S.D.

Compound	0	2	Time (h) 4	6	12
			(cpm/3 dishes) x 10^{-5}		
Cell					
PE	85.9±3.15	66.2±5.07	56.3±1.40	44.9±5.9	29.7±3.8
PC	9.11±1.02	25.9±4.0	31.6±2.6	31.3±2.1	29.4±0.4
Glycerol phospho-ethanolaime	3.84±0.75	6.00±0.47	10.5±2.2	9.77±0.48	9.87±0.54
Ethanolamine	1.14±0.13	0.87±0.17	1.37±0.47	1.57±0.45	2.88±1.57
Ethanolamine-phosphate	4.27±0.80	3.38±0.28	8.42±2.85	8.35±2.12	11.7±2.6
Medium					
PE	0	0.45±0.14	0.52±0.06	0.81±0.10	1.17±0.31
PC	0	0.17±0.06	0.35±0.09	0.79±0.07	1.60±0.20
Glycerol phospho-ethanolaime	0	1.28±0.22	1.44±0.22	3.23±0.39	9.01±1.02
Ethanolamine	0	0.76±0.07	0.24±0.02	2.46±0.25	4.85±0.49
Ethanolamine-phosphate	0	0.95±0.17	0.72±0.10	2.06±0.37	3.77±1.35

TABLE II.3.

PERCENT DISTRIBUTION OF [^{3}H]ETHANOLAMINE-LABELED PE MOLECULAR SPECIES IN HEPATOCYTES AS A FUNCTION OF TIME

Primary hepatocytes were cultured for 12-18 h in Dulbecco's MEM containing 17% fetal bovine serum. At the start of the experiment medium was replaced with fresh MEM and incubated for 30 min. The medium was then replaced with 2 ml MEM containing 7.5 uCi/ml [1-^{3}H]ethanolamine and incubated for 1 h. At the end of the pulse the medium was replaced with MEM containing 100 µM ethanolamine. At the indicated times PE was isolated from 3 dishes of cells as described in the Methods. PE was fractionated into molecular species using reverse phase HPLC. The percent distribution of radioactivity and lipid phosphorus of each peak was determined. Results are expressed as the mean of 3 separate pulse-chase experiments ±S.D.

Molecular Species	Time (h)						
	0	2	6	12	18	24	mol% cellular PE
16:1-18:2, 18:2-22:6	0.8±0.5	0.4±0.2	0.6±0.3	0.3±0.2	0.5±0.3	0.5±0.1	0.5±0.3
18:2-20:4, 18:2-18:2	0.7±0.1	0.9±0.5	0.7±0.4	0.3±0.1	0.5±0.1	0.5±0.2	0.8±0.8
16:0-22:6 (I)	44.5±8.4	35.4±10.0	25.4±5.6	18.2±3.0	19.1±0.6	15.0±2.3	11.5±2.3
16:0-20:4 (II)	11.2±4.4	14.9±4.8	14.8±4.2	11.7±4.6	13.1±5.9	9.2±2.7	6.9±1.3
16:0-18:2, 18:1-18:2 (III)	6.5±2.2	7.0±2.5	6.0±1.7	5.1±1.4	4.6±1.3	4.4±1.1	4.0±1.2
18:0-20:5, 16:0-22:5	2.8±0.5	2.3±0.6	2.6±1.0	2.2±0.5	2.1±0.1	2.0±0.5	2.4±1.1
18:0-22:6 (IV)	13.3±3.6	15.6±4.1	19.2±3.7	22.1±3.3	15.3±1.1	20.4±5.8	15.6±3.5
18:0-20:4 (V)	15.6±3.4	18.5±3.6	24.8±3.3	35.3±4.4	38.6±3.2	44.1±3.9	50.9±5.8
18:0-18:2, 18:0-22:5 (VI)	3.3±1.7	3.8±2.2	4.5±2.9	3.5±1.5	3.0±1.3	3.1±1.3	4.4±1.5
18:0-20:3, 18:0-22:5	0.5±0.1	0.6±0.2	0.8±0.4	0.7±0.4	1.0±0.1	0.9±0.4	1.9±1.0

TABLE II.4.

TURNOVER TIMES AND TURNOVER RATES OF PE MOLECULAR SPECIES

Turnover times and turnover rates were calculated according to the method of Zilversmit et al. (1948). Briefly, the turnover time (T_t) was derived using the expression T_t=1.44$t_{1/2}$, where $t_{1/2}$ is the half life of radioactivity in the individual molecular species measured from the data in Fig. III.7. Since the decay of radioactivity from each species is bimodal, two $t_{1/2}$ values were obtained, one between 0 h and 12 h (p1) and a one between 12 h and 24 h (p2). The pool size (r) for the individual species is the average from three experiments. The turnover rate (p) was derived from the expression p = r/T_t.

Molecular Species	Turnover Time (h) T_{t1}	T_{t2}	r (nmol/10^7cells)	Turnover rate (nmol/h) p1	p2
16:0-22:6 (I)	5.4	32.7	31.6	5.8	1.0
16:0-20:4 (II)	12.2	30.5	23.9	2.0	0.8
16:0-18:2, 18:1-18:2 (III)	8.9	34.2	16.6	1.9	0.5
18:0-22:6 (IV)	18.5	36.3	36.8	2.0	1.0
18:0-20:4 (V)	32.2	81.8	95.0	3.0	1.2
18:0-18:2, 18:0-22:5 (VI)	12.1	35.7	18.2	1.5	0.5
Total PE	10.2	58.7	222.1	21.9	3.8

TABLE II.5

PERCENT DISTRIBUTION OF [^{3}H]ETHANOLAMINE-LABELED PC MOLECULAR SPECIES IN HEPATOCYTES AS A FUNCTION OF TIME

PC was labeled from [1-^{3}H]ethanolamine and isolated as described in Table II.2. The molecular species were separated by HPLC. The results are expressed as the mean of 4 separate pulse-chase experiments ± S.D.

Molecular Species	0	2	6	12	18	24	mol% cellular PC
16:1-18:2, 18:2-22:6	0.7±0.2	0.7±0.3	0.5±0.2	0.8±0.1	0.4±0.4	0.8±0.4	1.8±1.1
18:2-20:4, 18:2-18:2	1.1±0.1	0.9±0.1	0.9±0.2	0.8±0.2	0.7±0.4	0.7±0.2	2.5±0.1
16:0-22:6 (I)	37.5±6.5	29.0±3.9	19.8±7.5	9.2±3.1	7.2±2.7	6.2±2.1	6.3±1.1
16:0-20:4 (II)	15.7±3.6	20.9±6.6	21.7±5.6	20.6±5.1	20.8±2.9	19.3±4.2	13.2±0.9
16:0-18:2, 18:1-18-18:2(III)	7.2±1.2	8.2±0.9	8.3±0.9	8.8±1.0	9.2±2.0	10.2±2.9	10.4±1.5
18:0-20:5, 16:0-22:5	2.6±1.0	2.3±0.2	2.0±0.5	1.6±0.3	1.7±0.2	1.5±0.2	2.9±0.4
18:0-22:6 (IV)	17.0±4.0	17.2±3.4	18.2±4.1	16.9±3.7	17.8±5.8	16.7±5.9	11.9±0.9
18:0-20:4 (V)	10.9±3.1	16.1±2.0	22.8±7.2	34.7±8.4	34.8±11.2	42.2±7.9	31.3±1.9
18:0-18:2, 18:0-22:5 (VI)	2.5±0.8	4.0±1.7	4.8±1.7	5.9±0.6	6.4±1.6	6.9±1.4	10.3±1.8
18:0-20:3, 18:0-22:5	0.7±0.2	0.7±0.1	0.8±0.4	0.7±0.5	1.4±0.6	0.8±0.1	2.4±0.1

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CHAPTER III.

Role of PEMT in the Metabolism of PE and PC[1]

Introduction

As discussed in section I, hepatic PE can be synthesized by at least three separate pathways: 1) the CDP-ethanolamine pathway (Kennedy and Weiss, 1956); 2) the decarboxylation of phosphatidylserine (PS) (Hübscher et al., 1959); and 3) the reaction of PS synthase (Bjerve, 1973). While the relative contribution of each pathway to PE synthesis is unknown, several investigations have suggested that the CDP-ethanolamine pathway is the major route of PE biosynthesis (Bell and Coleman, 1980; van den Bosch, 1974; Tijburg et al., 1989; Zelinski and Choy, 19824). The bulk of PE synthesis from the CDP-ethanolamine pathway occurs on the endoplasmic reticulum, though the Golgi also has the capacity to make PE by this pathway (Vance, J. E. and Vance, D. E., 1988). Unlike the PC made by the CDP-choline pathway, the PE made by the CDP-ethanolamine pathway does not undergo any significant remodeling of the acyl chains (Samborski et al., 1990).

In the liver, PE is converted to PC by the enzyme PE-*N*-methyltransferase (PEMT), an 18.3 kdal integral membrane protein (Ridgway and Vance, 1987). The composition of newly-made PC, derived from the PE, is enriched in docosahexaenoate-containing species compared to newly-made PC synthesized from CDP-choline (Samborski et al., 1990). PC synthesis by the methylation pathway might contribute 20 to 40% of the total PC in liver (Sundler and Åkesson, 1975).

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The possibility that the methylation pathway provides a hexaenoic-rich fraction of the PC in the liver (Glen and Austin, 1971) and synpatosomes (Tacconi and Wurtman, 1985) has been previously suggested but remains unproven. A clear function of PEMT in the liver is the production of choline (Vance and Ridgway, 1986). Thus, while PE methylation can provide an auxillary source of PC in the liver, it is not clear if this is the primary function of PEMT.

Newly-made PE, rather than the bulk of cellular PE, is the preferential substrate for PEMT *in vivo* (Samborski et al., 1990). If not methylated the newly made PE is rapidly degraded by a phospholipase to glycerolphosphoethanolamine (GPE) and other catabolites (Samborski et al., 1990). In particular, 16:0-22:6-PE had a turnover rate (5.8 nmol/h/10^7 cells) which was two or more times that of any other major molecular species of PE (Samborski et al., 1990). The nature and locations of the phospholipases involved in PE catabolism is unknown but the possibility of the existance of PE selective phospholipases can not be dismissed. Recently, there have been several reports on the presence of an arachidonate-selective phospholipase A_2 in polymorphonuclear leukocytes (van den Bosch et al., 1990; Schalkwijk et al., 1990) and in a monocytic leukemic cell line (Diez and Mong, 1990; Clark et al., 1990). Thus, hepatic PE homeostasis must involve the coordinate regulation of three synthetic pathways and at least two degradative pathway. With respect to PE catabolism it is not clear if cellular phospholipases and PEMT compete for newly-synthesized PE or utilize separate pools. Currently, nothing is known about the possible *in vivo* interaction of cellular phospholipases and PEMT.

To study further the catabolic fate of hepatic PE, made by the CDP-ethanolamine pathway, we have used the analogue of adenosine, 3-deazaadenosine (DZA), to inhibit the PEMT catalysed conversion of PE to PC (Pritchard et al., 1982). DZA is converted to 3-deazaadenosylhomocysteine, a strong inhibitor of *S*-adenosylhomocysteine hydrolase which causes a rise in the cellular levels of *S*-adenosylhomocysteine and decreases the levels of *S*-adenosylmethionine in rat hepatocytes (Chiang and Cantoni, 1979). PEMT is especially sensitive to the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine (Hoffman et al., 1980) and is therefore rapidly inhibited by DZA-treatment (Pritchard et al., 1982).

We now report that prolonged inhibition of PEMT by DZA did not alter the molecular species composition of PE or PC in rat hepatocytes. Moreover, the overall catabolism of PE was not affected by DZA treatment. However a major exception was 16:0-22:6-PE which, instead of being methylated to PC, was catabolized rapidly in DZA-treated cells. We also found that PC, derived from PE, was remodeled on both the *sn*-1 and *sn*-2 positions and this proccess was not affected by inhibition of the methyltransferase. The major species being remodeled was 16:0-22:6-PC. The rapid turnover of 16:0-22:6-PE and PC species compared to other PE and PC species may be due to the presence of a 16:0-22:6 selective phospholipase.

Materials and Methods

Materials

The same materials were used in the studies as shown in chapter II 3-Deazaadenosine (DZA) was a gift from Drs. Peter Chiang and Guilio Cantoni.

Animals and Hepatoctes

Same as described in chapter II.

Pulse chase experiments

Just prior to the start of the experiment the medium was removed and replaced with serum-free MEM and the cells were equilibriated for 30 min. Fifteen µCi of [1-^{3}H]ethanolamine was added to each dish in 2 ml serum-deficient MEM and the cells were incubated for 1 h. The medium was replaced with 2 ml of serum-deficient MEM containing either 100 µM ethanolamine or 100 µM ethanolamine and 10 µM DZA and the cells were subsequently incubated for the indicated times.

Experimental analysis

Same as described in chapter II with the following exceptions:

1) In some experiments the cells were sonicated prior to extraction of lipids and an aliquot was taken for protein determination (Lowry et al., 1951).

2) In some experiments phospholipids were visualized by exposure to iodine vapors. In these experiments PE and PC were scraped from the plate and analyzed for phosphorus and radioactivity.

3) Analyses of PE and PC molecular species were performed on a 4.6 x 250 mm Ultrasphere ODS column (Beckman) using a Perkin-Elmer liquid chromatograph.

RESULTS

Effects of DZA on PC and PE compostion

Cultured rat hepatocytes were incubated in serum-free medium containing 10 µM DZA for 0, 2, 4, 6, and 12 h. Fig. III.1 shows that the DZA-treatment caused a small but significant increase in cellular PE levels over control cells during 12 h of exposure ($p<0.0005$). However, during the first 4 h of DZA-treatment this effect was not significant ($0.05<p<.1$). Consistent with earlier reports (Pritchard et al., 1982; Vance et al., 1986) we observed that DZA did not significantly alter cellular PC levels (Fig. III.1.). PC and PE were isolated from the experiments depicted in Fig. III.1. and fractionated into molecular species by HPLC. Fig. III.2. shows that treatment of cells with 10 µM DZA for up to 12 h did not significantly alter the mol% distribution of the major PE molecular species. Extending these studies to 24 h showed a similar lack of effect. These results indicate that while the blockage of PE to PC conversion does cause a small elevation in cellular PE levels, the fatty acid composition of PE is not altered. Furthermore, the conversion of PE to PC is apparently not an important mechanism in maintaining cellular PE composition. Fig. III.3. shows that DZA did not signficantly alter the mol% distribution of the major PC molecular species. In particular, the cellular levels of 16:0-22:6-PC (species 7) and 18:0-22:6-PC (species 13) were unaffected by DZA-treatment. If PE methylation provides a specific, hexaenoic-rich, fraction of the cell PC (Trewhella and Collins, 1970; Glen and Austin, 1971; Tacconi and Wurtman, 1985) then prolonged inhibition of this pathway with 10 µM DZA should result in the depletion of this fraction of PC (species 7 and 13). This depletion did not occur. This result is consistent with our earlier observation that the CDP-choline pathway can provide all the major PC molecular species including the docosahexaenoate-rich species (Samborski et al., 1990). Taken together these results suggest that in hepatocytes PE methylation is not necessary for maintenance of either PE or PC molecular species composition.

Effects of DZA on the Catabolism of [^{3}H]Ethanolamine-Labeled PE.

Cultured rat hepatocytes were incubated for 1 h with [1-^{3}H]ethanolamine and chased for up to 12 h with medium containing 100 μM unlabeled ethanolamine ± 10 μM DZA. Fig. III.4. shows the incorporation of label into cellular PE and PC. The extensive decay of radioactivity from the PE pool of control cells during the chase period and the incorporation of [1-^{3}H]ethanolamine-labeled PE into PC in control cells, which reached a plateau between 2 and 4 h of the chase, is in agreement with our previous findings (Samborski et al., 1990). Fig. III.4. shows that in DZA-treated cells the conversion of [1-^{3}H]ethanolamine-labeled PE to PC during the initial 4 h of the chase was inhibited by 93% compared to controls. The corresponding decay of radioactivity from PE in DZA-treated cells was slower than in control cells. This difference could be completely accounted for by a lack of conversion of PE to PC during the initial 4 h of the chase. During the 4-12-h period of the chase similar amounts of radiolabel decayed from the PE pool in control and DZA-treated cell (73.3 x 10^5dpm and 67.2 x 10^5dpm, respectively). Using the method of Zilversmit et al. (1948), the turnover rates of PE during the 4-12-h period of the chase in control and DZA-treated cells were calculated to be 18.3 and 15.6 nmole/h/10^7 cells, respectively.

The labeling of PC in control and DZA-treated cells is also shown in Fig. III.4. During the 4-12-h period of the chase 5.85 x 10^5 dpm of the [1-^{3}H]ethanolamine-derived label was lost from the PC pool in control cells while during the same period 4.80 x 10^5 dpm decayed from the PC pool in DZA-treated cells (Fig. III.4.). These values are approximately 10-fold less than that for the decay of label from the corresponding PE species. These results indicate that the turnover of [1-^{3}H]ethanolamine-labeled PC is much slower than the turnover of [1-^{3}H]ethanolamine-labeled PE and that the turnover of PC is not dependent on the activity of PEMT.

To determine the nature of the PE catabolites, we analyzed the organic and aqueous components from both the cells and medium of the experiments depicted in Fig. III.4. The data for control cells is shown in Table III.1. During the first 2 h of the chase approximately 78% of the label lost from PE was converted into PC. During the 4-12-h chase period, the label lost from PE was mostly accounted for by an increase in cell and medium [1-^{3}H]ethanolamine-labeled GPE, ethanolamine-phosphate, and ethanolamine. Approximately 53% of the radiolabel lost from PE was secreted into the medium, the major catabolite being GPE. Loss of radiolabel due to PE secretion accounted for 2%.

The data for DZA-treated cells is shown in Table III.2. During the first 4 h of the chase approximately 17% of the label lost from PE was converted into PC reflecting the time for DZA to enter the cell and inhibit PEMT. As with control cells, the remaining label lost from PE was accounted for by an increase in cell and medium [1-^{3}H]ethanolamine-labeled GPE, ethanolamine-phosphate, and ethanolamine (Table III.2.). During the 4-12-h chase period the rate of label lost from PE in DZA-treated cells was similar to that seen for control cells (Table III.1.). Interestingly, in the presence or absence of DZA the same amount of PE-derived label appeared in the aqueous catabolites. This result suggests that a specific portion of the newly-synthesized PE is targeted for methylation while another portion is targeted for degradation by phospholipases. Blocking the methylation pathway does not cause more PE to be channeled towards these unknown phospholipases as might be expected if PEMT and the phospholipases compete for the same substrate. In summary, it would appear that the phospholipase catalyzed degradation of PE occurs irrespective of PE methylation and inhibition of PEMT does not re-route bulk PE towards the degradation pathway.

Effects of DZA on the Metabolism of PE Molecular Species

The [1-^{3}H]ethanolamine-labeled PE, isolated from the experiments depicted in Fig. III.4., was fractionated into molecular species by HPLC. The percent distribution of label in PE molecular species of control and DZA-treated cells is shown in Table III.3. In agreement with earlier studies (Samborski et al., 1990; Åkesson et al., 1976; Åkesson et al., 1970; Trewhella and Collins, 1973; Åkesson, 1970; Holub and Kuksis, 1971) which described the major labeled molecular species as a hexaenoate-rich fraction, labeled 16:0-22:6-PE at the end of the pulse accounted for 41% of the incorporation of [1-^{3}H]ethanolamine into PE. In Table III.3. a comparison of the data for control and DZA-treated cells indicated that there were no significant differences in % radioactivity in any of the major PE species during the 12 h chase.

From measurements of the masses and radioactivity of the major PE molecular species we were able to calculate specific activities. Figs. III.5. and III.6. shows that DZA-treatment had different effects on the catabolism of various PE species. Fig. III.5

A shows that the inhibition of PEMT did not significantly impair the decay of radiolabel from 16:0-22:6-PE during the initial 2 h of the chase. We calculated that the turnover rates for 16:0-22:6-PE during the 0-12-h chase period in control and DZA-treated cells were 4.8 and 4.3 nmole/h/10^7 cells, respectively. Figs. III.5. and III.6. (panels B-D) show that DZA-treatment retarded the decay of radiolabel from 16:0-20:4-, 16:0-18:2-, and 18:0-22:6-PE. During the 0-12-h chase period the turnover rates of 16:0-20:4-, 16:0-18:2-, and 18:0-22:6-PE in control and DZA-treated cells were 1.8, 1.1, 2.9 nmole/h/10^7 cell and 1.0, 0.7, 1.6 nmole/h/10^7 cells, respectively. Fig. III.6. (panels E and F) show that DZA-treatment resulted in a small increase in the specific activity of 18:0-20:4-PE and 18:0-18:2-PE after 12 h of exposure.

The results presented in panels B, C and D of Figs. III.5. and III.6. were similar to those seen in Fig. III.4. The delay in decay of radioactivity reflects the lack of conversion of PE to PC during the first 2 h and no apparent increase in PE catabolism. After 2 h, within experimental error, the rate of catabolism of PE is similar in the control and DZA-treated hepatocytes. In contrast, the 16:0-22:6-PE, which would normally have been methylated to PC in control cells, was apparently rapidly degraded in DZA-treated cells. One interpretation of this result is that if 16:0-22:6-PE is not methylated, a phospholipase immediately degrades this species of PE. In panels E and F of Fig. III.6. there is an indication of some impairment of PE degradation in DZA-treated cells.

Effects of DZA on the Remodeling of ethanolamine-derived PC

As mentioned earlier, the major metabolic fate of PE during the early part of the experiments depicted in Fig. III.4. is the conversion to PC. Thus, PC was isolated from the experiments described in Fig. III.4. and fractionated into molecular species by HPLC. From measurements of the masses and the radioactivity of the major PC molecular species we were able to calculate the specific activity of [1-^{3}H]ethanolamine-labeled PC.

Fig. III.7. and III.8. shows the results for control and DZA-treated cells, respectively. Fig. III.7. indicates that the 16:0-22:6-PC was the major labeled species at the end of the pulse. As with the specific activity of total PC (Fig. III.4.), the specific activity of all molecular species increased during the 2-4-h period of the chase (Fig. III.7.). However, during the 4-12-h chase period the static appearance of the specific activity of total PC (Fig. III.4.) masked the remodeling of some PC species that was occurring. This remodeling was obscured during the 0-4-h chase period by the continued synthesis of PC from PE. During the 4-12-h chase period 16:0-22:6-PC was being remodeled into other PC species, predominantly 18:0-20:4-PC. During this period the turnover rate of 16:0-22:6-PC was calculated to be 4.3 nmole/h/10^7 cells, a value very similar to the turnover rate calculated for 16:0-22:6-PE.

DZA-treatment of cells allowed for the examination of remodeling in newly-synthesized PC species in the absence of any further synthesis (Fig. III.8.). During the 0-4-h chase period the specific activity of 16:0-22:6-PC decreased by greater than 30% while the specific activity of most of the other major species either increased or remained unchanged. As PC synthesis by PE methylation had been blocked, the increases in specific activity in PC molecular species must have been due to the remodeling of existing PC species. The data in Figs. III.7 and III.8. indicate that there is a rapid remodeling of 16:0-22:6-PC after its synthesis. This result suggests that newly-made PC, derived from PE, is immediately accessible to PC-remodeling enzymes. Remodeling of PC continued to occur during the 4-12-h chase period with losses of radioactivity from 16:0-22:6-PC accounting for 68% of the radioactivity appearing in 18:0-20:4-, 18:0-18:2- and 18:0-22:5-PC. During the 0-4-h period the turnover rate of 16:0-22:6-PC was calculated to be 8.6 nmole/h/10^7 cells. During the 4-12-h period the turnover rate of 16:0-22:6-PC was calculated to be 6.3 nmole/h/10^7 cells. These results indicate that remodeling of PC occurs irrespective of its synthesis from PE and that both newly-synthesized and older PC are subjected to deacylation/reacylation reactions. Clearly, 16:0-22:6-PC is the major substrate remodeled in these experiments with molecular species having stearate in the *sn*-1 position and predominantly either 20:4 or 18:2 in the *sn*-2 position being the major final products.

DISCUSSION

PEMT is not Required for Maintainence of Cellular PE and PC Composition

The methylation of PE to PC can account for a significant portion of PC production in the hepatocyte (Sundler and Åkesson, 1975). If the methylation pathway were critically important in the generation of a specific pool of PC, inhibition of this pathway might result in the depletion of this PC pool and a concomitant increase in the corresponding PE pool. We have now demonstrated that the prolonged inhibition of cellular PEMT, with 10 µM DZA, did not affect the mol% distribution of either PE or PC molecular species (Figs. III.2. and III.3.). The hexaenoic-rich PC fraction (species 7 and 13) comprised approximately 16.4% of the total PC mass at time 0 h and after 12 h of incubation in the presence or absence of DZA comprized 15.4 and 15.3%, respectively. This result is consistent with our previous finding that the rat hepatocyte can synthesize significant amounts of hexaenoic-rich PC via the CDP-choline pathway (Samborski et al., 1990). It would thus appear, that contrary to previous ideas (Glen and Austin, 1971; Tacconi and Wurtman, 1985) PE methylation is not required for the maintainence of a particular molecular species pool of PC. Another liver function where PE methylation is not essential is lipoprotein secretion by cultured rat hepatocytes (Vance J. E. et al., 1986).

Selective Degradation of 16:0-22:6-PE

Previously we suggested that newly-made PE would flow from the vicinity of the PEMT and encounter phospholipases in a separate subcellular compartment (Samborski et al., 1990). However, the precise site of PE degradation is not known and phospholipases are present in most, if not all, subcellular compartments (Waite, 1987). It is known that rat liver microsomes contain the bulk of cell PEMT and primarily phospholipase A_1 and a lysophospholipase (Vance and Vance, 1988; Holub, 1982; Pind et al., 1985). If PEMT and phospholipases compete for the same pool of newly-made PE we would have expected that inhibition of PEMT with DZA would result in an increased catabolism of PE by phospholipases. This was not the case for the bulk of PE as the data in Tables III.1. and III.2. indicate that essentially the same amount of PE was degraded to glycerolphosphoethanolamine, ethanolamine-phosphate, and ethanolamine in both control and DZA-treated cells.

However, when individual molecular species of PE were analyzed, DZA did affect the fate of certain species. Most notable is the apparent lack of effect of PEMT inhibition on the decay of radiolabel from 16:0-22:6-PE. In control cells substantial amounts of 16:0-22:6-PE is converted to PC during the initial 2 h of the chase (Fig. III.7.) compared to that degraded by phospholipases. In DZA-treated cells there is a similar decay of radiolabel from 16:0-22:6-PE during this same period that can only be attributed to degradation or remodeling. Compared to total PE there appears to be re-routing of 16:0-22:6-PE from the methylation pathway to the degradation pathway.

In contrast to 16:0-22:6-PE, the catabolism of 16:0-20:4-, 16:0-18:2- and 18:0-22:6-PE was unaffected by DZA. The results suggest a competition between PEMT and phospholipase for only the 16:0-22:6-PE species. Is it possible that PEMT occupies the same compartment as a 16:0-22:6-specific phospholipase? The existence of phospholipase A_2 with selective activity on phospholipids that contain 20:4 is well documented in several types of cells (see Chapter I: section III.A.II). However, the existence of 20:4 specific phospholipase A_2 remains to be proven. A major criticism by van den Bosch et al. (1990) is that the acyl-chain specificities of cellular phospholipase A_2 determined *in vitro* may reflect substrate packing densities and not actual specificities. While we agree with this criticism of *in vitro* studies, it does not apply to acyl-chain specificities determined by *in vivo* analysis, where the substrate is heterogeneous. In agreement with this is the recent report from Tessner et al. (1990) of the selective deacylation of 20:4-containing PE in stimulated human neutrophils. In addition, Holbrook and Wurtman (1990) have described a phospholipase D activity in rat synaptosomes that has a remarkable specificity for 22:6-containing phosphlipids. Taken together, the existence of a 16:0-22:6-selective phospholipase seems plausible.

What might be the purpose of a 16:0-22:6 selective phospholipase? Tinco et al. (1970) have sugested that part of the PE, containing the 22:6 moiety, is being recycled. As our results indicate that glycerolphosphoethanolamine is the major PE breakdown product, we propose that the 22:6 moiety is selectively being removed from the 16:0-22:6-PE, resynthesized into 16:0-22:6-diacylglycerol and re-utilized by the ethanolaminephosphotransferase reaction. Such a mechanism would be consistent with the observed rapid turnover of the head group of 16:0-22:6-PE and a hypothesized slower turnover of the 22:6 moiety. This proposal also provides a plausible explanation for the conservation of the docosahexanoate during essential fatty acid deficiency in rats (Yamamoto et al., 1965; Lyman et al., 1969).

In DZA-treated cells there was a small increase in the specific activity of 18:0-20:4-PE during the chase period, with the greatest change occurring during the first 2 h ($p<0.1$). This may reflect a decrease in catabolism of this molecular species (Pind et al., 1985; Kuksis et al., 1975). Alternatively, this increase may reflect new synthesis of this species through remodeling of 16:0-22:6-PE and other species. From the data in Tables III.2. and III.3. we have calculated that during the initial 4 h of chase there is an increase of 6.97×10^5 dpm in 18:0-20:4-PE while there is a loss of 39.73 x 10^5 dpm from 16:0-22:6-PE. If we assume that the increase in 18:0-20:4-PE radioactivity were due to remodeling, this increase would represent 18% of the radiolabel lost from 16:0-22:6-PE during this period. During the 4 to 12 h chase period 31.39 x 10^5 dpm are lost from 16:0-22:6-PE, of which less than 1% could have appeared in 18:0-20:4-PE. Thus, in control hepatocytes there is no apparent remodeling of PE species (Samborski et al., 1990), possibly due to the presence of PEMT. In DZA-treated cells some remodeling of PE molecular species may have occurred but this would only apply to newly-made PE during the first few hours.

The endoplasmic reticulum is usually considered the primary site of PE synthesis, although the Golgi and a fraction associated with mitochondria also have enzymes of the CDP-ethanolamine pathway (Vance and Vance, 1988; Vance, J. E., 1990). PE synthesized in the mitochondria by the PS-decarboxylase pathway is metabolically distinct from PE synthesized by the CDP-ethanolamine pathway (Vance, J. E., 1989). While the majority of serine-derived PE is synthesized in the mitochondria (Voelker, 1984; Kuge et al., 1986) the role the mitochondria has in the metabolism of ethanolamine-derived PE remains to be determined. We do not know if ethanolamine-derived PE, made on the ER, is metabolically distinct from ethanolamine-derived PE made in the Golgi or mitochondrial-fraction X complex (Vance, J. E., 1990).

PE turnover is much faster in cultured rat hepatocytes (Samborski et al., 1990) compared to Chinese hamster ovary cell cultures (Miller and Kent, 1986), bovine aortic endothelial cells (Lipton et al., 1990) and HeLa cells (unpublished results), cell lines that express little PEMT activity. Does PE-N-methyltransferase have a role in modulating the phospholipase catalyzed degradation of PE? The results of this study demonstrate that the degradation of PE is not dependent on the activity of PEMT as the decay of radioactivity from PE is similar in both control and DZA-treated cells during the 4-12-h chase period. During this period we have calculated that PE turnover in the presence or absence of DZA is 18.3 and 15.6 nmol/h/10^7 cells, respectively.

PC is Rapidly Remodeled at Both the *sn*-1 and *sn*-2 Positions

While there is extensive remodeling of PC molecular species it is usually thought to be limited to the *sn*-2 position (Lands and Crawford, 1976). We have recently shown that PC, synthesized from CDP-choline, is remodeled at both the *sn*-1 and *sn*-2 positions in hepatocytes derived from choline deficient rats (Tijburg et al. 1991).

While our earlier studies suggested that [1-^{3}H]ethanolamine labeled PC was remodeled, the exact nature of this remodeling was obscured by continued synthesis of PC from PE (Samborski et al., 1990). We now demonstrate that [1-^{3}H]ethanolamine-labeled PC is remodeled at both the *sn*-1 and *sn*-2 positions immediately after synthesis in normal rat hepatocytes treated with DZA. Thus, contrary to current dogma remodeling of the *sn*-1 position of PC is an extremely significant reaction that contributes to the maintenance of cellular PC composition. While much of the current interest in phospholipases has centered on their roles in signal transduction via the generation of inositolphosphates and diacylglycerols, the important role these enzymes have in the maintenance of membrane composition has not been emphasized.

An important finding to emerge from these studies is that the 16:0-22:6-PC pool appears to be turning over rapidly. The turnover rate of [1-^{3}H]ethanolamine-labeled 16:0-22:6-PC was 4.3-6.3 nmole/h/10^7, a rate similar to that calculated for 16:0-22:6-PE. In another study we have calculated that in choline-deficient rat hepatocytes, the turnover rate of [methyl-^{3}H]choline-labeled 16:0-22:6-PC was 7.3 nmole/h/10^7 (Tijburg et al. 1991). These turnover rates are consistent with the rapid turnover of [methyl-^{3}H]methionine-labeled hexaenoic-PC in rat liver (Ridgway and Vance, 1988) and brain (Lakher and Wurtman, 1987). Clearly PE methylation is not required for the maintenance of the 16:0-22:6-PC pool as this species is rapidly metabolized upon synthesis from either choline or ethanolamine. Why should the cell expend energy on the synthesis of 16:0-22:6-PC and then quickly metabolize it? Is 16:0-22:6-PC an intermediate in the production of other PC species? We now present a hypothetical model that incorporates PEMT, dososaheaxenoate and the putative 16:0-22:6-selective phospholipase in the production of PC in hepatocytes (Fig. III.9.). In this model free 22:6 is incorporated into the 16:0-22:6-diacylglycerol (steps 1-3) and then subsequently used for the biosynthesis of 16:0-22:6-PE (step 4). If this species were not methylated to PC, it could be degraded to lysoPE by a 16:0-22:6-selective phospholipase A_2 (step 5) and then further to glycerol-phosphoethanolamine by a lysophospholipase (step 6). In this model we indicate a phospholipase A_2 in step 5. This is speculation and we do not rule out the possibility of a phospholipase A_1 in this step. Alternatively, 16:0-22:6-PE could be degraded by a phospholipase C to phosphoethanolamine and 16:0-22:6-diacylglycerol (step 7). Not shown in this figure is the combined action of PLD and PAP on PE resulting in the 16:0-22:6-diacylglycerol. The phosphoethanolamine might be secreted or further catabolized to ethanolamine (step 8) while the diacylglycerol could be re-utilized in step 4. 16:0-22:6-PC, derived from both PE methylation (step 9) and the CDP-choline pathway (step 10), could be remodeled at both the SN-1 and SN-2 positions to produce 18:0-20:4-PC (step 11). The 22:6 acyl groups, released during steps 6 and 11, could be recycled to 16:0-22:6-diacylglycerol for another round of PE and PC biosynthesis (steps 12, 1-3). Ultimately, PC synthesized from the methylation pathway would be broken down and the choline moiety released into the cell. In times of choline deficiency this pathway would become important in the maintenance of choline levels in animals. In support of this model, the CDP-ethanolamine pathway in the liver synthesizes PE with a molecular composition enriched in 22:6 (greater than 40 mol%), while the bulk of the cell PE contains much less 22:6 (10.3-12.4 mol%; ref. Samborski et al., 1990; Holub and Kuksis, 1971; Tinco et al., 1970; Lyman et al., 1973). The selectivity of the CDP-ethanolamine pathway for 22:6 has been attributed to the specificity of the CDP-ethanolamine: 1,2-diacylglycerol ethanolaminephosphotransferase for the 16:0-22:6-diacylglycerol (Holub, 1977; Kanoh, 1969). The selectivity of the ethanolaminephosphotransferase for 16:0-22:6-diacylglycerol seems remarkable as rat liver diacylglycerol contains 0.6-1.7 mol% of the 16:0-22:6 species (Åkesson, 1969). This observation has led Tinco et al. to speculate that there may be another, more important, precursor for generation of 16:0-22:6-diacylglycerol (Tinco et al., 1970). Potential candidates for this donor role are the corresponding 16:0-22:6-containing PC and PE species.

FIGURE III.1.

10 μM DZA SLIGHTLY INCREASES THE MASS OF CELLULAR PE, BUT NOT PC, IN HEPATOCYTES.

Cultured rat hepatocytes were incubated in the presence or absence of DZA (10 μM) for up to 12 h. Dishes were harvested and cell protein and cell PE and PC levels were determined as described under Materials and Methods. Results are the mean ± S.E.M. of 14-18 determinations.

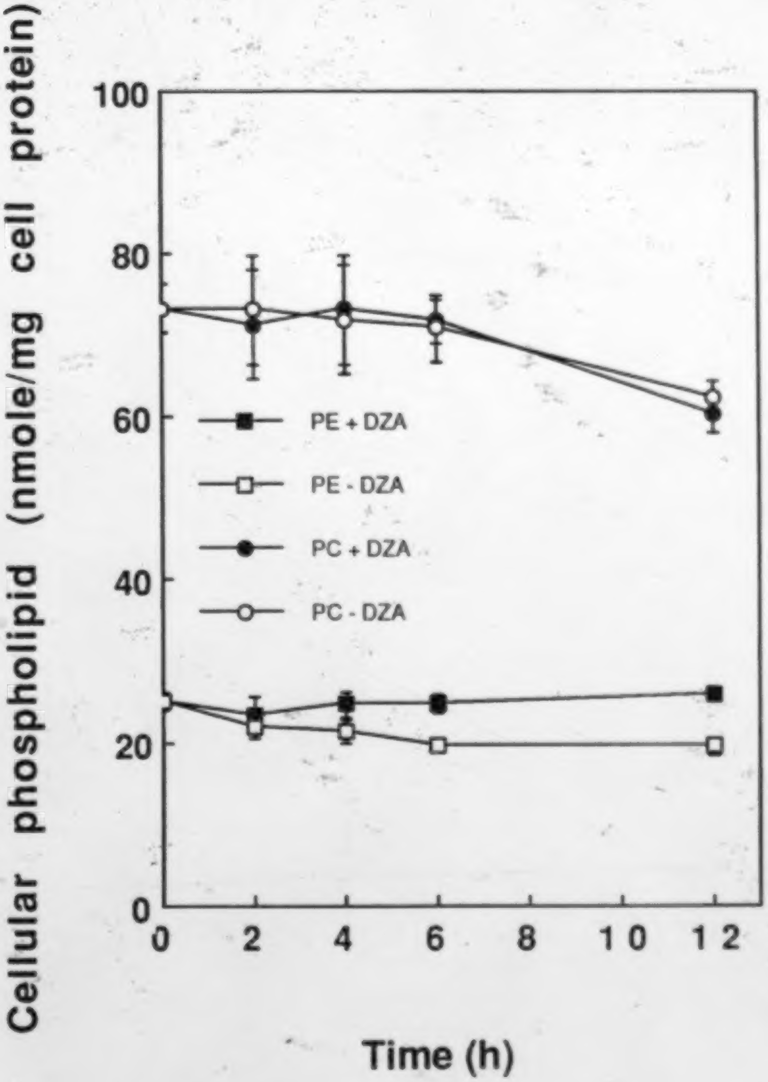
Cellular phospholipid (nmole/mg cell protein)
100
80
60
40
20
0
0
2
4
6
8
10
12
Time (h)
PE + DZA
PE - DZA
PC + DZA
PC - DZA

FIGURE III.2.

10 µM DZA DOES NOT ALTER THE MAJOR MOLECULAR SPECIES OF CELLULAR PE.

PE, isolated from the experiments depicted in Fig. 1, were fractionated into molecular species by HPLC and then analyzed for phosphorus content as described under Materials and Methods. Values are the mean of four separate experiments ± S.E.M. The numbers on the x axis refer to the molecular species (7, 16:0-22:6; 8, 16:0-20:4; 9/10, 16:0-18:2 & 18:1-18:2; 11, 18:0-20:5 & 16:0-22:5; 13, 18:0-22:6; 14, 18:0-20:4; 15/16 18:0-18:2 & 18:0-22:5).

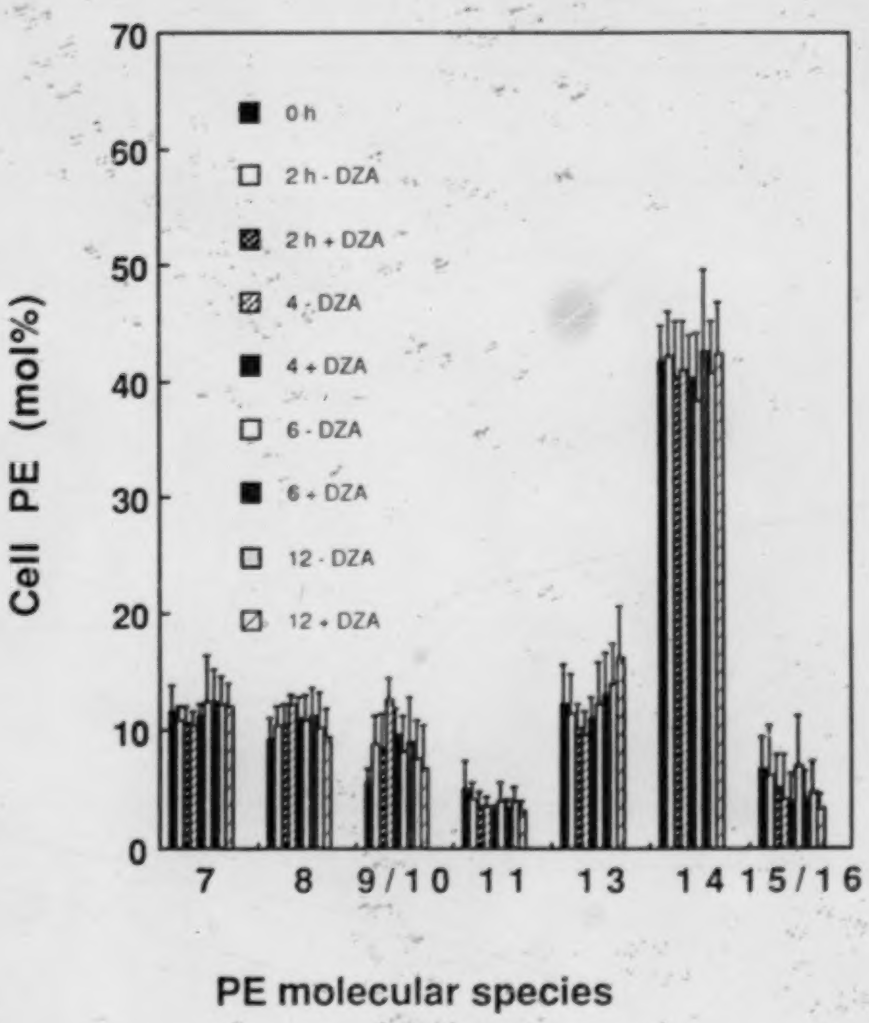
70
60
50
40
30
20
10
0
Cell PE (mol%)
0 h
2 h - DZA
2 h + DZA
4 - DZA
4 + DZA
6 - DZA
6 + DZA
12 - DZA
12 + DZA
7
8
9/10
11
13
14
15/16
PE molecular species

FIGURE III.3.

10 µM DZA DOES NOT ALTER THE MAJOR MOLECULAR SPECIES OF CELLULAR PC.

PC, isolated from the experiments depicted in Fig. 1, were fractionated into molecular species by HPLC and then analyzed for phosphorus content as described under Materials and Methods. Values are the mean of four separate experiments ± S.E.M. The numbers on the x axis refer to the molecular species (7, 16:0-22:6; 8, 16:0-20:4; 9/10, 16:0-18:2 & 18:1-18:2; 11, 18:0-20:5 & 16:0-22:5; 12, 16:0-18:1; 13, 18:0-22:6; 14, 18:0-20:4; 15/16 18:0-18:2 & 18:0-22:5).

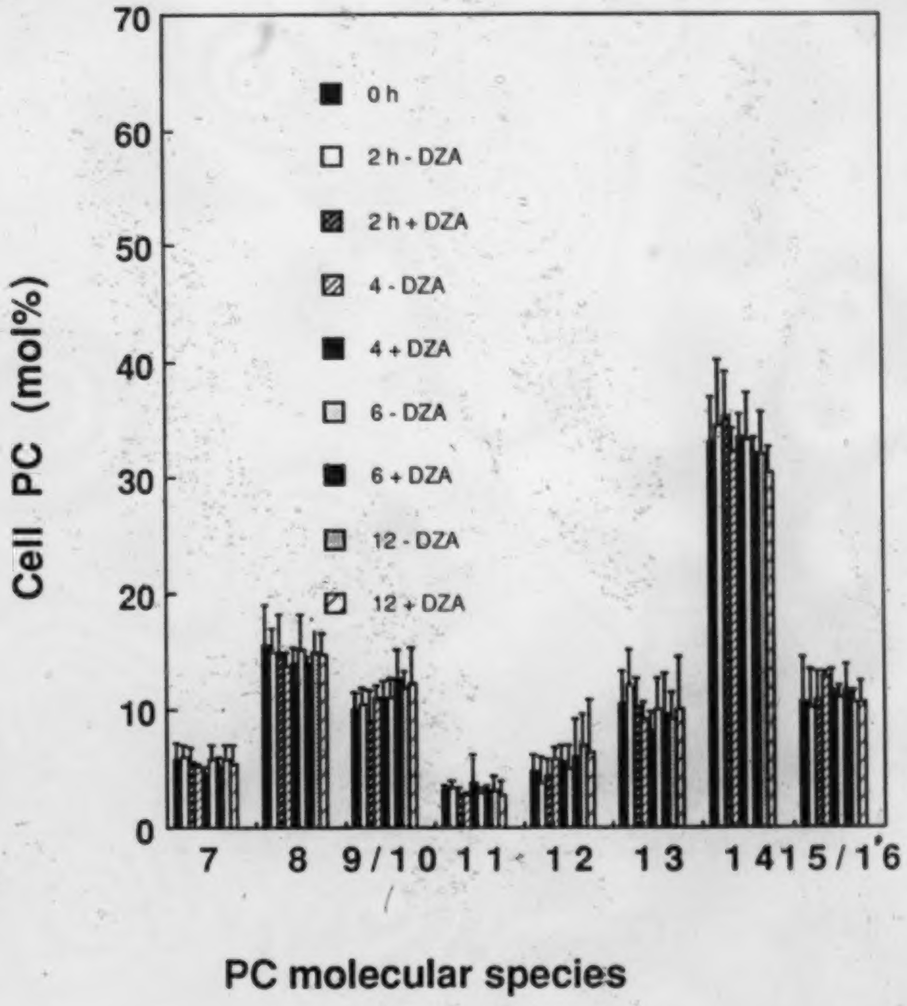
Cell PC (mol%)
0
10
20
30
40
50
60
70
0 h
2 h - DZA
2 h + DZA
4 - DZA
4 + DZA
6 - DZA
6 + DZA
12 - DZA
12 + DZA
7
8
9/10
11
12
13
14
15/16
PC molecular species

FIGURE III.4.

EFFECT OF 10 μM DZA ON THE TOTAL RADIOACTIVITY OF CELLULAR PE AND PC DERIVED FROM [^{3}H]ETHANOLAMINE.

Primary hepatocytes were cultured for 12-18 h in Dulbecco's MEM containing 17% fetal bovine serum. At the start of the experiment medium was replaced with fresh MEM without serum and incubated for 30 min. The medium was replaced with 2 ml MEM containing 7.5 μCi/ml [1-^{3}H]ethanolamine and incubated for 1 h. At the end of the pulse the medium was replaced with MEM containing either 100 μM ethanolamine or 100 μM ethanolamine and 10 μM DZA. At the indicated times the PE and PC were isolated from 3 dishes of cells as described in the Materials and Methods. Results are expressed as the mean of 3 separate pulse-chase experiments ± S.D.

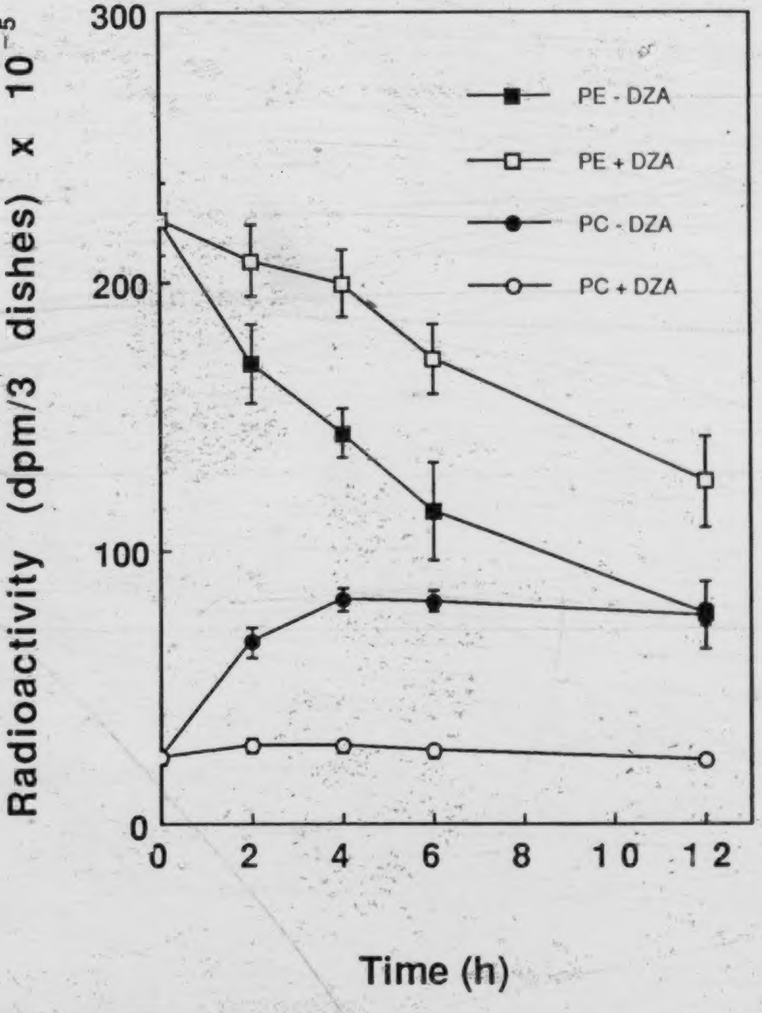
Radioactivity (dpm/3 dishes) x 10^{-5}
300
200
100
0
0
2
4
6
8
10
12
Time (h)
PE - DZA
PE + DZA
PC - DZA
PC + DZA

FIGURE III.5.

EFFECTS OF 10 μM DZA ON THE SPECIFIC RADIOACTIVITY OF MOLECULAR SPECIES OF CELLULAR PE DERIVED FROM [^{3}H]ETHANOLAMINE. (PART 1)

PE, isolated from the experiments depicted in Fig III.4., was fractionated into molecular species by HPLC and then analyzed for radioactivity and phosphorus content as described under Materials and Methods. The major PE molecular species presented are: 16:0-22:6, panel A; 16:0-20:4, panel B; 16:0-18:2, panel C; 18:0-22:6. The results for control cells are shown in open symbols and the results for DZA-treated cells are shown in solid symbols. Values are the mean of three separate experiments ± S.E.M.

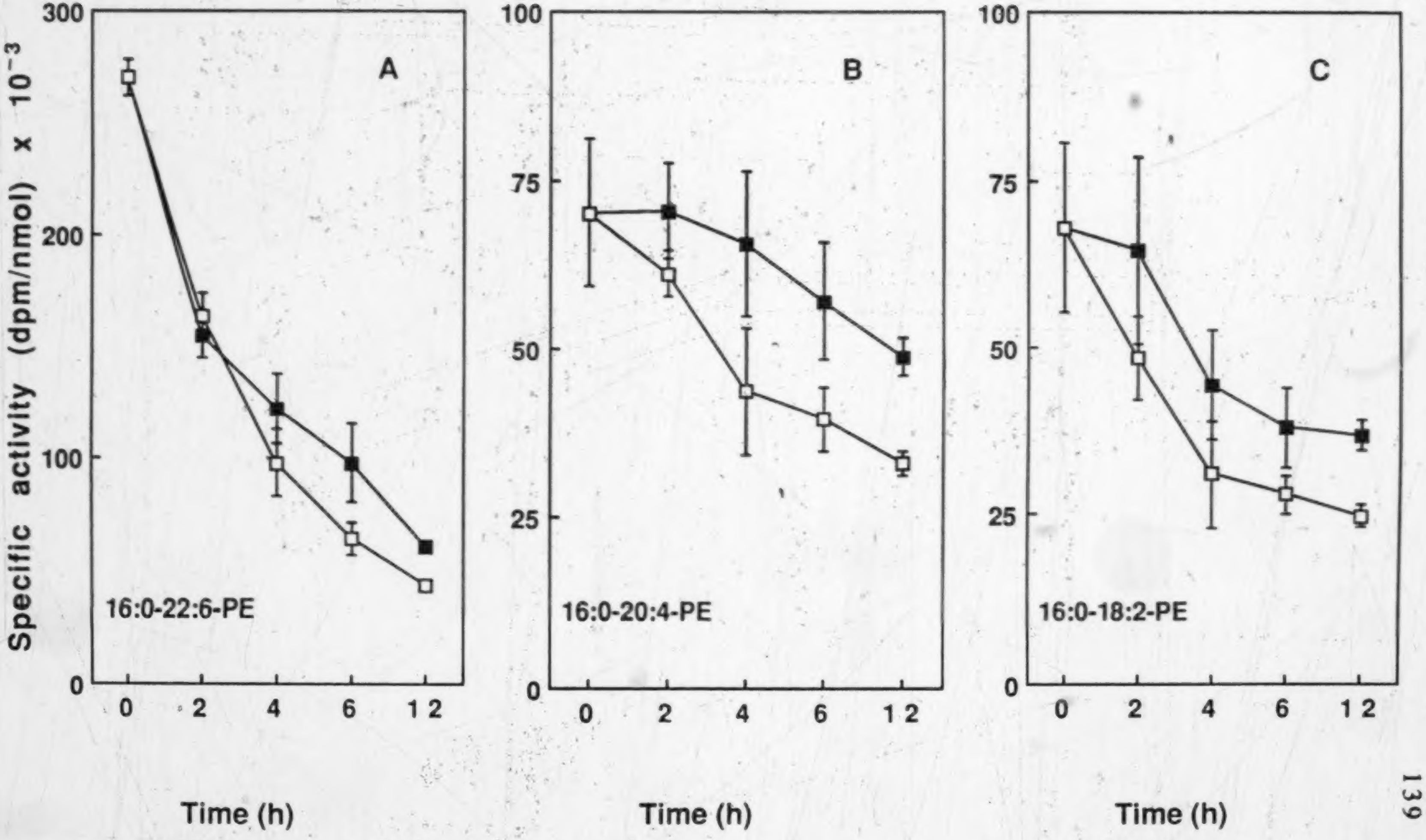
Specific activity (dpm/nmol) x 10⁻³
300
200
100
0
A
16:0-22:6-PE
0
2
4
6
1 2
Time (h)
100
75
50
25
0
B
16:0-20:4-PE
0
2
4
6
1 2
Time (h)
100
75
50
25
0
C
16:0-18:2-PE
0
2
4
6
1 2
Time (h)

FIGURE III.6.

EFFECTS OF 10 μM DZA ON THE SPECIFIC RADIOACTIVITY OF MOLECULAR SPECIES OF CELLULAR PE DERIVED FROM [^{3}H]ETHANOLAMINE. (PART 2)

PE, isolated from the experiments depicted in Fig III.4., was fractionated into molecular species by HPLC and then analyzed for radioactivity and phosphorus content as described under Materials and Methods. The major PE molecular species presented are: 18:0-22:6, panel D; 18:0-20:4, panel E; and 18:0-18:2, panel F. The results for control cells are shown in open symbols and the results for DZA-treated cells are shown in solid symbols. Values are the mean of three separate experiments ± S.E.M.

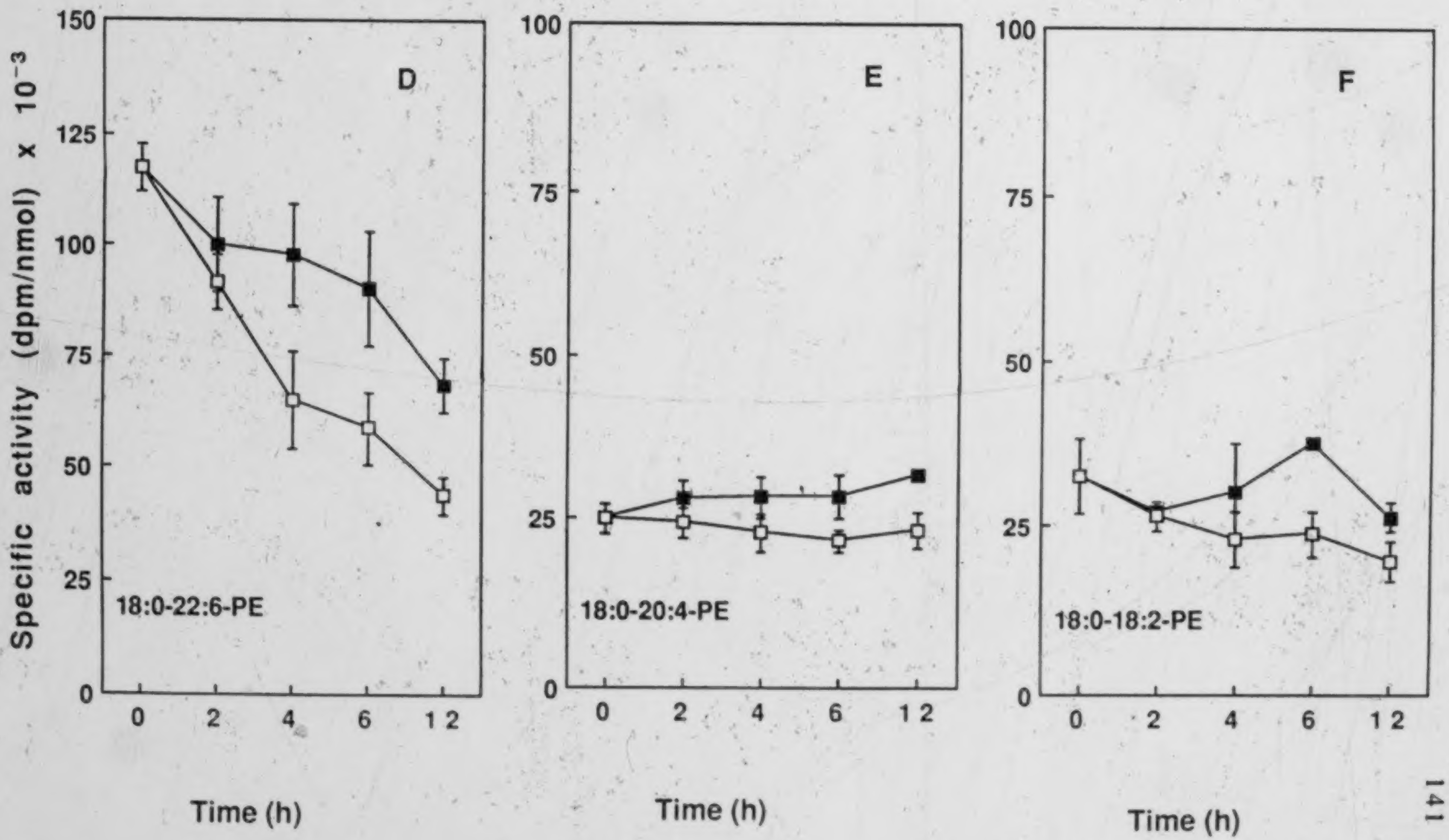
Specific activity (dpm/nmol) x 10^{-3}
150
125
100
75
50
25
0
D
18:0-22:6-PE
0
2
4
6
1 2
Time (h)
100
75
50
25
0
E
18:0-20:4-PE
0
2
4
6
1 2
Time (h)
100
75
50
25
0
F
18:0-18:2-PE
0
2
4
6
1 2
Time (h)

FIGURE III.7.

EFFECT OF 10 μM DZA ON THE SPECIFIC RADIOACTIVITY OF MOLECULAR SPECIES OF CELLULAR PC DERIVED FROM [^{3}H]ETHANOLAMINE.

PC, isolated from the experiments depicted in Fig. III.4., were fractionated into molecular species by HPLC and analyzed for radioactivity and phosphorus content as described under Materials and Methods. The results for control cells are shown. Values are the mean of three separate experiments ± S.D. The number on the x axis refer to the molecular species (7, 16:0-22:6; 8, 16:0-20:4; 9, 16:0-18:2; 10, 18:1-18:2; 11, 18:0-20:5 & 16:0-22:5; 12, 16:0-18:1; 13, 18:0-22:6; 14, 18:0-20:4; 15, 18:0-18:2; and 16, 18:0-22:5).

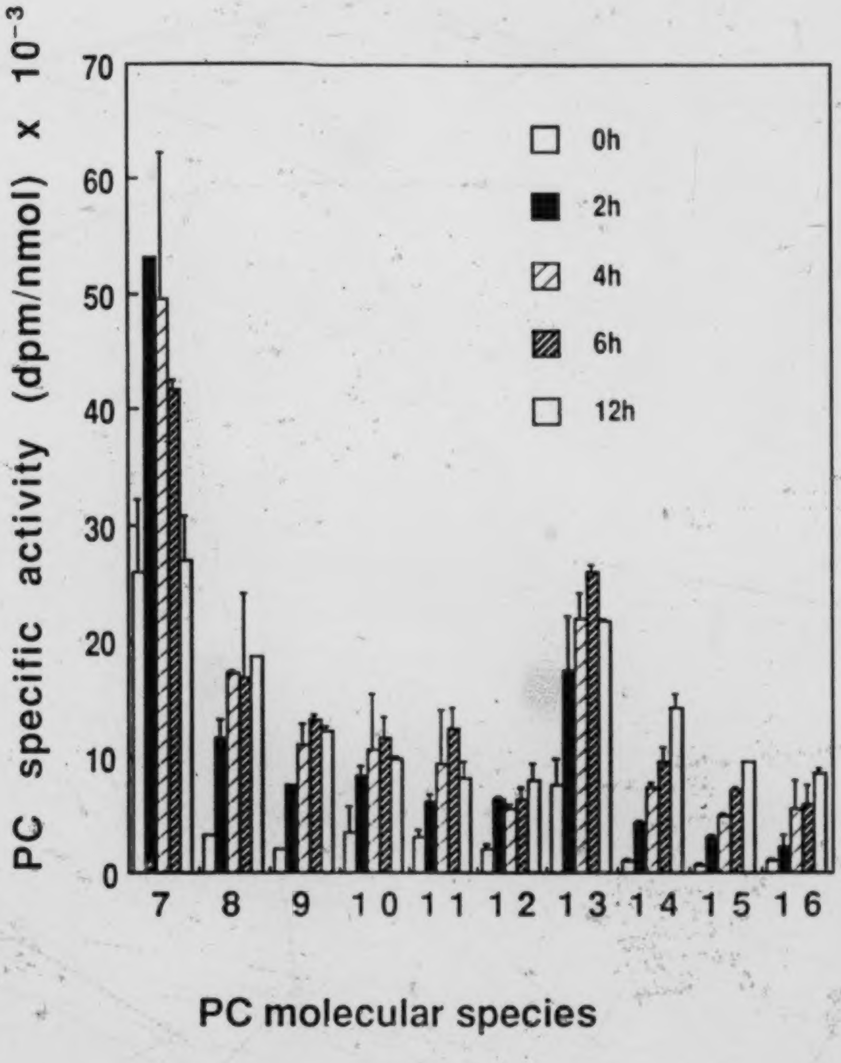
PC specific activity (dpm/nmol) x 10^{-3}
0
10
20
30
40
50
60
70
☐ 0h
■ 2h
▨ 4h
▨ 6h
☐ 12h
7
8
9
10
11
12
13
14
15
16
PC molecular species

FIGURE III.8.

EFFECT OF 10 μM DZA ON THE SPECIFIC RADIOACTIVITY OF MOLECULAR SPECIES OF CELLULAR PC DERIVED FROM [^{3}H]ETHANOLAMINE.

PC, isolated from the experiments depicted in Fig. III.4., were fractionated into molecular species by HPLC and analyzed for radioactivity and phosphorus content as described under Materials and Methods. The results for DZA treated cells are shown. Values are the mean of three separate experiments ± S.D. The number on the x axis refer to the molecular species (7, 16:0-22:6; 8, 16:0-20:4; 9, 16:0-18:2; 10, 18:1-18:2; 11, 18:0-20:5 & 16:0-22:5; 12, 16:0-18:1; 13, 18:0-22:6; 14, 18:0-20:4; 15, 18:0-18:2; and 16, 18:0-22:5).

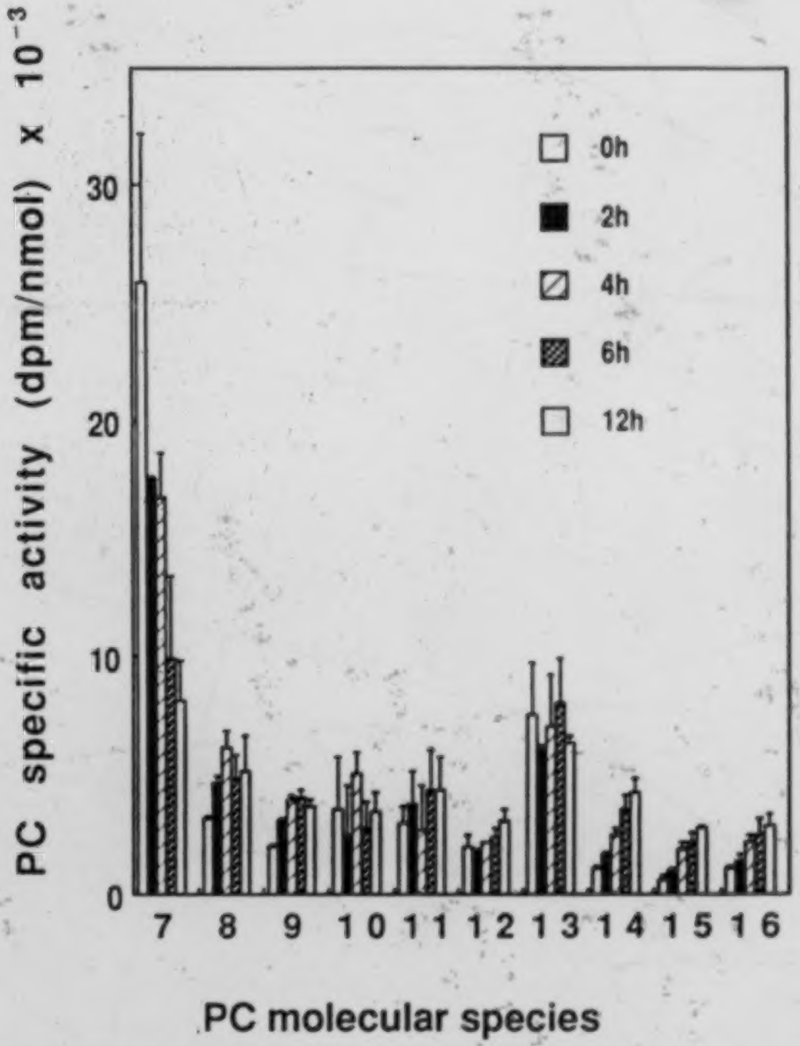
PC specific activity (dpm/nmol) x 10^{-3}
0
10
20
30
☐ 0h
■ 2h
▨ 4h
▩ 6h
☐ 12h
7
8
9
10
11
12
13
14
15
16
PC molecular species

FIGURE III.9.

FIG. 7. AN HYPOTHETICAL DOCOSAHEXAENOATE CYCLE INVOLVED IN THE BIOSYNTHESIS OF PC.

1) biosynthesis of 22:6-CoA by acyl-CoA synthetase; 2) biosynthesis of 16:0-22:6-phosphatidic acid by 1-acylglycerol-3-P acyltransferae; 3) biosynthesis of 16:0-22:6-diacylglycerol by phosphatidic acid phosphatase; 4) biosynthesis of 16:0-22:6-phosphatidylethanolamine (PE) by the enzyme CDP-ethanolamine: 1,2-diacylglycerol ethanolaminephosphotransferase, 5) selective deacylation of 16:0-22:6-PE by a phospholipase A and release of 22:6 acyl group; 6) degradation of 16:0-lyso PE by a lysophospholipase; 7) release of phosphoethanolamine (P-Etn) from 16:0-22:6-PE by a phospholipase C; 8) generation of ethanolamine from P-Etn; 9) conversion of newly-made 16:0-22:6-PE to 16:0-22:6-phosphatidylcholine (PC) by the enzyme PE-N-methyltransferase; 10) biosynthesis of 16:0-22:6-PC by the enzyme CDP-choline:1,2-diacylglycerol cholinephosphotransferase; 11) remodeling of 16:0-22:6-PC at both the SN-1 and SN-2 positions to form 18:0-20:4-PC; and 12) recycling of 22:6 acyl group for another round of PE and PC synthesis.

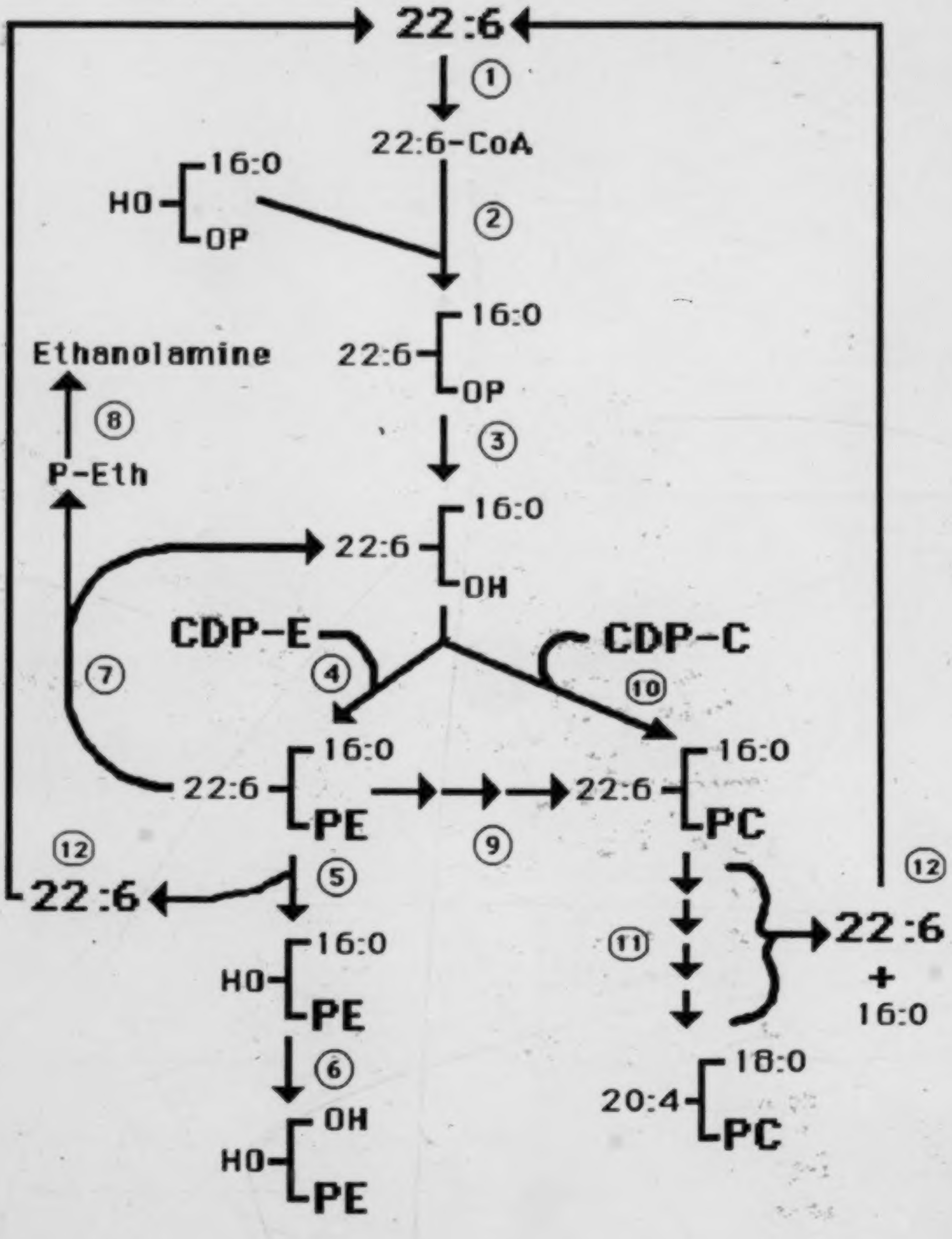
22:6
①
22:6-CoA
HO
16:0
OP
②
22:6
16:0
OP
Ethanolamine
⑧
P-Eth
③
22:6
16:0
OH
CDP-E
④
⑦
CDP-C
⑩
22:6
16:0
PE
⑨
22:6
16:0
PC
⑫
22:6
⑤
HO
16:0
PE
⑥
HO
OH
PE
⑪
22:6
+
16:0
⑫
20:4
18:0
PC

TABLE III.1.

CATABOLISM OF [1-^{3}H]ETHANOLAMINE-LABELED PE DURING A 12 HOUR CHASE IN THE ABSENCE OF 10 μM DZA

Primary hepatocytes were cultured for 12-18 h in Dulbecco's MEM containing 17% fetal bovine serum. At the start of the experiment medium was replaced with fresh MEM without serum and incubated for 30 min. The medium was replaced with 2 ml MEM containing 7.5 uCi/ml [1-^{3}H]ethanolamine and incubated for 1 h. At the end of the pulse the medium was replaced with MEM containing 100 μM ethanolamine. At the indicated times the major PE catabolites were isolated from the medium and cells as described in the Methods. Results are expressed as the mean of 3 separate pulse-chase experiments ± S.D.

PE catabolite	Time (h) 0	2	4	6	12
			(dpm/3 dishes) x 10^{-5}		
Cell					
PE	223.2 ± 13.30	169.3 ± 14.7	143.9 ± 9.47	115.0 ± 18.0	76.8 ± 12.5
PC	24.0 ± 2.31	66.3 ± 5.48	82.0 ± 4.13	81.4 ± 3.76	76.9 ± 3.90
Glycerol Phospho-ethanolamine	9.8 ± 2.30	14.9 ± 1.70	26.6 ± 5.69	24.8 ± 1.88	25.4 ± 2.62
Ethanolamine	2.75 ± 0.65	1.91 ± 0.17	3.03 ± 0.79	3.57 ± 0.94	6.41 ± 3.60
Ethanolamine-Phosphate	11.1 ± 2.75	8.59 ± 0.19	20.0 ± 5.52	20.4 ± 4.97	29.5 ± 6.14
Medium					
PE	0	1.17 ± 0.39	1.35 ± 0.17	2.05 ± 0.21	2.85 ± 0.62
PC	0	0.46 ± 0.16	0.92 ± 0.25	2.03 ± 0.24	4.10 ± 0.29
Glycerol Phospho-ethanolamine	0	2.73 ± 0.76	5.47 ± 1.29	9.08 ± 0.47	22.8 ± 2.24
Ethanolamine	0	1.48 ± 0.76	4.93 ± 2.0	7.84 ± 2.47	13,4 ± 4.58
Ethanolamine-Phosphate	0	2.13 ± 0.76	2.56 ± 0.63	4.67 ± 0.72	7.66 ± 2.55

TABLE III.2.

CATABOLISM OF [1-^{3}H]ETHANOLAMINE-LABELED PE DURING A 12 HOUR CHASE IN THE PRESENCE OF 10 µM DZA

Primary hepatocytes were cultured for 12-18 h in Dulbecco's MEM containing 17% fetal bovine serum. At the start of the experiment medium was replaced with fresh MEM without serum and incbated for 30 min. The medium was replaced with 2 ml MEM containing 7.5 µCi/ml [1-^{3}H]-ethanolamine and incubated for 1 h. At the end of the pulse the medium was replaced with MEM containing 100 µM ethanolamine and 10 µM DZA. At the indicated times the major PE catabolites were isolated from the medium and cells as described in the Methods. Results are expressed as the mean of 3 separate pulse-chase experiments ± S.D.

PE catabolite	time (h) 0	2	4	6	12
		(dpm/3 dishes) x 10^{-5}			
Cell					
PE	223.2 ± 13.3	208.0 ± 13.7	199.2 ±12.8	171.6 ± 13.0	125.9 ± 16.8
PC	24.00 ± 2.31	28.9 ± 2.52	28.1 ± 2.48	26.5 ± 2.00	23.3 ± 0.83
Glycerol Phospho-ethanolamine	9.8 ± 2.30	14.7 ± 1.48	24.0 ± 3.75	26.3 ± 2.90	38.1 ± 6.18
Ethanolamine	2.75 ± 0.65	1.80 ± 0.26	2.91 ± 0.97	2.65 ± 0.32	5.83 ± 2.03
Ethanolamine-Phosphate	11.11 ± 2.75	7.32 ± 1.24	14.8 ± 2.88	17.1 ± 1.97	36.7 ± 8.07
Medium					
PE	0	1.32 ± 0.42	1.48 ± 0.23	2.35 ± 0.38	3.57 ± 0.67
PC	0	0.32 ± 0.09	0.47 ± 0.08	0.68 ± 0.01	1.23 ± 0.13
Glycerol Phospho-ethanolamine	0	2.76 ± 0.59	5.74 ± 0.97	9.78 ± 1.74	26.1 ± 2.34
Ethanolamine	0	2.26 ± 0.7	8.06 ± 4.93	7.01 ± 2.04	7.95 ± 4.99
Ethanolamine-Phosphate	0	1.83 ± 0.49	2.53 ± 0.68	3.97 ± 1.33	10.05 ± 2.65

TABLE III.3.

METABOLISM OF [1-^{3}H]ETHANOLAMINE-LABELED PE MOLECULAR SPECIES IN CONTROL AND DZA-TREATED HEPATOCYTES

Hepatocytes were pulsed for 60 min with 15 µCi/dish [1-^{3}H]ethanolamine. The pulse medium was removed, the monolayers were washed and fresh medium containing 100 µM ethanolamine ± 10 µM DZA was added. At the indicated times the cells were harvested and PE was isolated. After further fractionation by HPLC the amount of radioactivity and lipid phosphorus associated with each peak was determined. Data are the mean ± SD of 4 separate experiments.

molecular species	time (h)								
	0	2	2	4	4	6	6	12	12
	control	control	DZA	control	DZA	control	DZA	control	DZA
	% total dpm ± SD								
18:2-18:3, 14:0-22:6	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 01	0.5 ± 0.2	0.3 ± 0.1
16:1-18:2, 18:2-22:6	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.1
18:2-20:4, 18:2-18:2	1.0 ± 0.1	1.0 ± 0.7	1.0 ± 0.4	0.8 ± 0.4	1.0 ± 0.5	0.9 ± 0.3	1.0 ± 0.5	1.1 ± 0.6	0.7 ± 0.3
16:0-22:6	41.0 ± 1.4	30.6 ± 5.6	28.1 ± 4.9	24.7 ± 4.6	26.0 ± 1.3	21.8 ± 0.6	22.2 ± 1.6	16.2 ±1.7	16.2 ± 3.2
16:0-20:4	9.5 ± 0.8	12.7 ± 0.8	13.6 ± 2.3	14.4 ± 0.6	15.2 ± 0.7	14.4 ± 0.9	15.0 ± 1.7	13.4± 2.2	11.9 ± 2.3
16:0-18:2	3.7 ± 1.1	5.3 ± 0.9	6.5 ± 1.3	7.2 ± 0.6	6.4 ± 0.4	7.2 ± 0.6	6.5 ± 0.9	6.5 ± 0.9	5.5 ± 1.1
18:1-18:2	2.1 ± 1.1	2.1 ± 0.2	1.7 ± 0.2	1.9 ± 0.6	2.2 ± 0.7	1.9 ± 0.6	2.3 ± 0.6	2.4 ± 0.6	1.9 ± 0.5
18:0-20:5, 16:0-22:5	4.0 ± 1.5	2.6 ± 0.1	2.2 ± 0.1	2.6 ± 0.6	2.4 ± 0.1	3.2 ± 0.2	2.8 ± 0.4	3.0 ± 0.6	2.5 ± 0.5
16:0-18:1	2.8 ± 0.8	1.8 ± 0.2	1.7 ± 0.3	1.3 ± 0.3	1.0 ± 0.3	1.5 ± 0.1	0.9 ± 0.3	1.2 ± 0.5	0.7 ± 0.4
18:0-22:6	17.6 ± 2.0	17.3 ± 2.4	18.5 ± 1.2	18.2 ± 0.8	20.2 ± 1.7	17.7 ± 1.3	19.9 ± 1.5	17.7 ± 1.8	23.1 ± 1.7
18:0-20:4	14.9 ± 0.4	18.9 ± 2.9	19.9 ± 6.5	26.5 ± 5.5	21.8 ± 1.7	27.0 ±1.9	25.8 ± 1.9	33.4 ± 0.3	34.3±1.0
18:0-18:2	1.8 ± 0.7	1.3 ± 0.5	1.3 ± 0.8	1.2 ± 0.6	1.9 ± 0.1	2.3 ± 0.3	1.9 ± 0.4	2.6 ± 0.5	1.8 ± 0.2
18:0-22:5,18:0-20:3	1.0 ± 0.1	1.1 ± 0.2	1.2 ± 0.4	0.7 ± 0.9	1.2 ± 0.2	1.2 ± 0.1	1.0 ± 0.2	1.6 ± 0.3	0.9 ± 0.1

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CHAPTER IV.

SUBCELLULAR CATABOLISM OF [^{3}H]ETHANOLAMINE-LABELED PE IN RAT LIVER

INTRODUCTION

Previously, we have demonstrated that newly-made PE, derived from CDP-ethanolamine, is the preferential substrate for PEMT *in vivo* (Samborski et al., 1990). If not methylated the newly made PE is rapidly degraded by a phospholipase to glycerolphosphoethanolamine (GPE) and other catabolites (Samborski et al., 1990). Analysis of PE molecular species indicated that 16:0-22:6-PE had a turnover rate which was two or more times that of any other major molecular species of PE (Samborski et al., 1990). When PE methylation was inhibited with the drug 3-deazaadenosine (DZA), the catabolism of 16:0-22:6-PE was enhanced, suggesting that PE N-methyltransferase (PEMT) and a phospholipase competed for the 16:0-22:6-PE. This phenomena was not seen for any other major PE species. There are several possible explanations for this observation. Firstly, there may exist an intracellular phospholipase which selectively degrades 16:0-22:6-PE. The existence of phospholipase A_2 with selective activity on phospholipids that contain 20:4 is well documented in several types of cells (Leslie et al. 1988; Wijkander and Sundler, 1989; Tessner et al., 1990). In addition, a phospholipase D activity in rat synaptosomes, that has a remarkable specificity for 22:6-containing phospholipids, has been described (Holbrook and Wurtman, 1990). Taken together, the existence of a 16:0-22:6-selective phospholipase seems plausible. Secondly, the preferred catabolism of 16:0-22:6-PE may reflect differences in accessibility of the newly-made PE species to a phospholipase. It has been demonstrated that the polyunsaturated species of PE are particularly enriched in the inner leaflet of the human erythrocyte (Hullin, et al., 1991). In trout brush-border membranes, 64% of PE and 69% of PS are located in the inner leaflet and are enriched in docosahexaenoic acid compared to the outer leaflet (Pelletier et al., 1987). It has been suggested that newly-made PE is initially in a compartment which is accessible to phospholipase attack and subsequently transferred to a second compartment which is not accessible to phospholipase attack (Valtersson and Dallner, 1982). Finally, a third possibility is that 16:0-22:6-PE is selectively transferred to an intracellular compartment which contains the phospholipase responsible for its degradation. While phospholipases are present in most, if not all, subcellular compartments (Waite, 1987), the location of the phospholipase(s) responsible for the degradation of ethanolamine-derived PE is unknown.

It is well documented that the rat liver microsomes have a very active phospholipase A_1 (PLA_1) activity (Scherophof et al., 1966; Newkirk and Waite, 1973; reviewed in: Waite, 1987). Holub (1982) has studied the specificity of endogenous microsomal PLA_1 and failed to demonstrate any selectivity for PE molecular species. The substrate in this study was microsomal membranes, which were pre-labeled *in vitro* with either [^{14}C]palmitoyl-CoA or [^{14}C]stearoyl-CoA and exogenous lysoPE. The possibility that the newly-made [^{14}C]PE had not undergone complete equilibrium with the endogenous microsomal PE was recognized by Pind et al. (1985). To overcome this potential source of error Pind et al. (1985) used deuterated water to label the microsomal PE molecular species *in vivo*. Incubation of these microsomal membranes, under conditions which activate the endogenous PLA_1, and subsequent analysis of the molecular species failed to demonstrate any acyl chain selectivity. Because the microsomal membranes were labeled over a twenty-hour period the labeled PE species were not reflective of newly-made PE, derived from ethanolamine. In light of these potential problems and our results in chapter II and III, we have decided to re-evaluate the specificity of the endogenous PLA activity in rat liver microsomes and mitochondria. The following are the results of a preliminary study.

MATERIALS AND METHODS

Materials and Animals

The same as in chapter II with the exception that Percoll was obtained from Pharmacia, Uppsula, Sweden and rats were fasted overnight.

Portal vein injection of [^{3}H]ethanolamine into the rat

Rats were lightly anesthetized with diethylether prior to intraperitoneal injection of 5 mg pentobarbitone/100 g body weight. The abdominal cavity was opened and the portal vein was exposed. The portal vein was clamped and 500 µCi of [^{3}H]ethanolamine was injected, using a 30G needle and a 1 ml syringe. Pressure was applied to the wound for approximately 1 min and then the clamp was removed and the abdominal cavity was covered with a damp paper towel. After 10 min, the liver was removed and subcellular fractions were isolated.

Isolation of subcellular fractions

The microsomal fraction (100,000 x g_{max} pellet) and mitochondrial fraction were isolated by the method of Jordi et al. (1989) as modified by Vance (1991). Briefly, the excised liver was weighed, cut into small pieces and then placed in 4 volumes of ice-cold isolation buffer containing 250 mM mannitol, 5 mM Hepes, 0.5 mM EGTA, and 0.1% BSA, pH 7.4 (buffer A). The liver was homogenized in a Potter-Elvehjem tube by four strokes of a loosely fitting pestle rotating at 650 rpm. Homogenates were centrifuged twice for 5 min at 600 x g. The supernatant was centrifuged for 15 min at 10,000 x g. The 10,000 x g pellet was suspended in 4 ml of buffer A and evenly distributed over 4 tubes, each containing 20 ml of 225 mM mannitol, 25 mM Hepes, 0.1 mM EGTA, 30% (v/v) Percoll and 0.1% BSA, pH 7.4. The suspension was centrifuged for 30 min at 95,000 x g and the lower band, consisting of mitochondria, was removed. The mitochondrial fraction was resuspended in buffer A and centrifuged twice for 5 min at 6,300 x g. The final pellet was resuspended in 3 ml of buffer A and 200 µl aliquots were stored at -70°C. The 10,000 x g supernatant was centrifuged for 60 min at 100,000 x g resulting in a microsomal pellet. The microsomes were re-solubilized in approximately 5 ml of buffer A and 200 µl aliquots were store until needed at -70°C. The determination of protein concentration and the specific activities of PE and PC has been described in chapter III. The purity of these membrane preparations has been described previously (Vance, 1991).

Endogenous phospholipase A assay

This assay is similar to the one used by Pind et al. (1985). Typically, 200 µl of either [^{3}H]ethanolamine-labeled microsomes or [^{3}H]ethanolamine-labeled mitochondria (containing 5.2 and 5.9 mg protein, respectively) were added to 1.0 ml of 50 mM Tris-HCl, pH 8.5, containing 10 mM $CaCl_2$. The samples were immediately placed in a 37°C shaking water bath (250 rpm) and incubated for the indicated times. The reaction was stopped by the addition of 4.8 ml of chloroform/methanol (2:1, v/v). The PE and PC were isolated by TLC, eluted from the silica gel, and stored at -20°C prior to analysis by HPLC.

RESULTS

500 µCi of [^{3}H]ethanolamine was injected into the portal vein of a 150 g female rat as described in Materials and Methods. After 10 min the liver was removed and the microsomal and mitochondrial fractions were isolated as previously described. Table IV.I. shows the characterization of these subcellular fractions. These results are similar to those previously reported (Vance, 1988). Previously, we have reported that there is preferential degradation of certain PE molecular species in rat hepatocytes (Samborski et al., 1990; chapter III). As both microsomes and mitochondrial fractions possess highly active phospholipase activity (Waite, 1987), we decided to study the *in vitro* catabolism of [^{3}H]ethanolamine-labeled PE in these subcellular fractions.

Catabolism of [^{3}H]ethanolamine-Labeled PE in rat liver microsomes

200 µl of [^{3}H]-labeled rat liver microsomes, containing 5.2 mg protein, were incubated from 0 to 60 min under conditions, described in Materials and Methods, which stimulate the endogenous PLA$_1$ activity. The results of a representative experiment is shown in Fig. IV.1. The linear decay of radiolabel from PE is consistent with earlier reports (Holub, 1982; Pind et al., 1985). The major lipid catabolite at the end of the 60 min incubation was lysoPE, which accounted for 27% of the total catabolites. The remaining 73% was accounted for by aqueous catabolites, ethanolamine, ethanolamine-phosphate (E-P), and glycerolphosphoethanolamine (GPE). Analysis of the aqueous catabolites by TLC indicated that, at the end of the 60 min incubation period, ethanolamine, E-P, and GPE accounted for 12%, 5%, and, 56% of the total catabolites, respectively. Interestingly, PC is apparently not catabolized under these conditions.

It is generally assumed that the catabolism of microsomal PE, under these experimental conditions, is the result of PLA$_1$ activity. This observation is supported by the finding that, in these experiments, GPE is the major catabolite. The presence of radiolabel in ethanolamine and E-P may arise via the further hydrolysis of GPE. Alternatively, radiolabeled E-P could be also generated by the action of PLC on PE, while radiolabeled ethanolamine could be generated by the action of PLD on PE. If GPE were being catabolized to E-P and ethanolamine in our microsome preparations, we should be able to demonstrate this by the inclusion of excess GPE and E-P in these incubations. The inclusion of 100 μM cold GPE or E-P in some incubations did not affect the final distribution of radiolabel in the aqueous catabolites (results not shown). These findings suggest that in addition to PLA_1 other phospholipases may be active during this incubations.

Microsomal PE, was fractionated into molecular species by HPLC (Table IV.2.). At the start of the incubation the molecular species labeled to the largest extent were 16:0-22:6-PE and 18:0-20:4-PE (Table IV.2.). By the end of the 60 min. incubation there was a slight but significant ($p<0.005$, $n=4$) decrease in the percent distribution of label in 16:0-22:6-PE. Concomitantly, there was a small but insignificant increase ($0.1<p<0.375$) in the percent distribution of radiolabel in 18:0-22:6-PE and 18:0-20:4-PE. The percent distribution of all other PE molecular species remained unchanged during the incubation.

From measurements of the masses of the major PE molecular species, we were able to calculate the specific activity of $[^3H]$ethanolamine-labeled microsomal PE (Figs. IV.2.). Fig. IV.2. indicates that the specific activity of all the major molecular species did not change significantly during the incubation ($p<0.375$, $n=4$). This result confirms the previous report that both newly-made and bulk PE are equally accessible to the microsomal PLA_1 (Pind et al., 1985). Expression of these data as either percent distribution radioactivity or as specific activity indicate no selectivity of the microsomal PLA_1 towards individual PE species. Thus, based on these studies and others (Holub, 1982; Pind et al., 1985) it appears that the microsomal PLA_1 is not selective for any PE species.

Catabolism of $[^3H]$ethanolamine-labeled PE in rat liver mitochondria

200 μl of $[^3H]$-labeled rat liver mitochondria, containing 5.9 mg protein, were incubated from 0 to 60 min under the same conditions as the microsomes The results of a representative experiment is shown in Fig. IV.3. The linear decay of radiolabel from PE is consistent with an earlier report (De Winter et al., 1982). The major lipid catabolite at the end of the 60 min incubation was lysoPE, which accounted for 33% of the total catabolites. The remaining 73% was accounted for by aqueous catabolites, ethanolamine, ethanolamine-phosphate (E-P), and glycerolphosphoethanolamine (GPE). Analysis of the aqueous catabolites by TLC

indicated that, at the end of the 60 min incubation, ethanolamine, E-P, and GPE accounted for 17%, 13%, and, 42% of the total catabolites, respectively. As in rat liver microsomes, PC is not catabolized under these conditions.

Mitochondrial PE, was fractionated into molecular species by HPLC (Table IV.3.). Similar to microsomal PE, at the start of the incubation the mitochondrial PE molecular species labeled to the largest extent were 16:0-22:6-PE and 18:0-20:4-PE (Table IV.3.). By the end of the 60 min incubation there was a slight but insignificant decrease ($0.1<p<0.375$, n=2) in the percent distribution of label in 16:0-22:6-PE. Concomitantly, there was a small but insignificant increase ($0.1<p<0.375$) in the percent distribution of radiolabel in 18:0-20:4-PE. The percent distribution of all other PE molecular species remained unchanged during the incubation.

From measurements of the masses of major PE molecular species, we were able to calculate the specific activity of [^{3}H]ethanolamine-labeled microsomal PE (Figs. IV.4.). Fig. IV.4. indicates that the specific activity of most of the major molecular species did not change significantly during the incubation ($p<0.375$, n=2). The specific radioactivity of 16:0-22:6-PE and 16:0-18:2-PE decreased during the incubation period by 36% and 64%, respectively. However, the decrease of the specific activity of 16:0-22:6-PE was not particularly significant ($0.1<p<0.375$, n=2) while the decrease seen for 16:0-18:2-PE was only marginally significant ($0.05<p<0.1$, n=2). Thus, it would appear that the PLA_2 associated with the mitochondrial fraction does not demonstrate any selectivity towards PE molecular species. This finding is consistent with the recent observation that PLA_2, from either human polymorphonuclear cell, human platelets or rat platelets, does not demonstrate acyl chain selectivity *in vitro* (Schalkwijk et al., 1990).

DISCUSSION

This study was undertaken to re-evaluate the specificity of the endogenous PLA activity in rat liver microsomes and mitochondria. Our preliminary findings are consistant with those previously reported (Holub, 1982; Pind et al., 1985; De Winter et al., 1982; Schalkwijk et al., 1990), and suggest that both microsomal associated PLA_1, and mitochondrial associated PLA_2, do not specifically degrade 16:0-22:6-PE *in vitro* . While these studies appear to rule out the involvement of these enzymes in the rapid and selective catabolism of 16:0-22:6-PE in the hepatocyte they must be viewed with some caution.

Firstly, it is important to stress that the lack of specificity *in vitro* does not necessarily reflect the actual *in vivo* specificity of these enzymes. PE is continually being synthesized on the cytosolic surface of the endoplasmic reticulum by the CDP-ethanolamine pathway (Bell et al., 1981). Potentially, newly-made 16:0-22:6-PE could insert more rapidly into the bilayer than other PE molecular species and thereby come in contact with the phospholipase sooner. De Winter et al. (1987) have demonstrated that the hydrolysis of PE by mitochondrial PLA_2 proceeds nearly entirely via intramembrane enzyme action (the PLA_2 acts on substrate in its own membrane and not in other membranes). Thus, the appearence of *in vivo* selectivity would simply reflect the individual rates of insertion into membranes for specific molecular species. During the course of cell fractionation, the newly-made PE molecules will have reached an equilibrium with the bulk of membrane PE (Figs. IV.2. and IV.4.) and the subsequent rate of PE hydrolysis would only reflect the catabolism of bulk PE. Alternatively, the insertion of newly-made 16:0-22:6-PE into the endoplasmic membrane will produce short-lived microdomains in the membrane which are enriched in the polyunsaturated species. Consistent with this hypothesis is the observation that greater than 50% of newly-made PE, derived from ethanolamine, is the 16:0-22:6 species. As membrane density is decreased by increasing the degree of unsaturation in the *sn*-2 fatty acid of PC (Demel et al., 1972) and since membrane packing can dramatically affect the penetration of a PLA_2 into the membrane (Demel et al., 1975), such local domains may give the appearance of in vivo selectivity. The fractionation of liver into microsomes and mitochondria probably eliminates such micro-domains.

Secondly, recent evidence suggests that mitochondrial associated PLA_2 may be an artefact of subcellular fractionation. Recently, work by Inada et al. (1991) have demonstrated that Kupffer cells contain 6-fold greater PLA_2 activity than hepatocytes. Furthermore, recent work by Wilton et al. (1991) suggests that the rat liver mitochondria PLA_2 is not actually present in the hepatocyte at all. Wilton et al. suggests that the "mitochondrial PLA_2" is derived from rat liver Kupffer cells and becomes associated with the mitochondrial fraction during isolation of the organelle. Possibily one of the recently discovered high molecular weight, Ca^{2+}-dependent intracellular PLA_2's (Loeb and Gross, 1986; Gronich et al., 1990; Wijkander and Sundler, 1989; Leslie et al. 1988; Diez and Mong, 1990; Clark et al., 1990) is involved in the catabolism of 16:0-22:6-PE in the hepatocyte.

Thirdly, it is not completely clear if the microsomal PLA_1 is an enzyme of the endoplasmic reticulum or simply a contaminant from some other subcellular membrane. Newkirk and Waite (1973) first demonstrated that the PLA_1 from rat liver plasma membranes and microsomes co-eluted during Sepharose 4b chromatography and both had a similar Ca^{2+} requirement. Waite and Sisson (1973) clearly demonstrated that rat liver plasma membranes was the source of heparin releasable PLA_1 in plasma. The membrane PLA_1 is thought to be involved in lipoprotein metabolism and is often referred to as hepatic lipase. This enzyme has been purified to homogeneity and shown to be a monomer with an apparent molecular weight of 57 kD (Kuusi et al., 1979; Jensen and Bensadoun, 1981). As microsomal preparation are usually contaminated with plasma membranes (Vance and Vance, 1988) the 'endogenous' PLA_1 activity we have been studying may actually be hepatic lipase. This point needs to be resolved. In addition, the potential role of a cytosolic PLA_1 in the catabolism of 16:0-22:6-PE can not be neglected. Unfortunately, these studies await the purification of cytosolic PLA_1.

FIGURE IV.1.

CATABOLISM OF MICROSOMAL [^{3}H]ETHANOLAMINE-LABELED PE BY ENDOGENOUS PHOSPHOLIPASE ACTIVTY.

Microsomes were prepared as described in the Materials and Methods. At the start of the experiment 200 µl of membranes were incubated with 1.0 ml of 50 mM Tris-HCl, pH 8.5 containing 10 mM $CaCl_2$. The samples were immediately placed in a 37°C shaking water bath (250 rpm) and incubated for the indicated times. The reaction was stopped by the addition of 4.8 ml of chloroform/methanol (2:1, v/v). The PC, PE and lysoPE were isolated by TLC and the radioactivity was determined as described in the Materials and Methods. The amount of radioactivity was determined on an aliquot of the aqueous phase (Aq). The results are of a representative experiment.

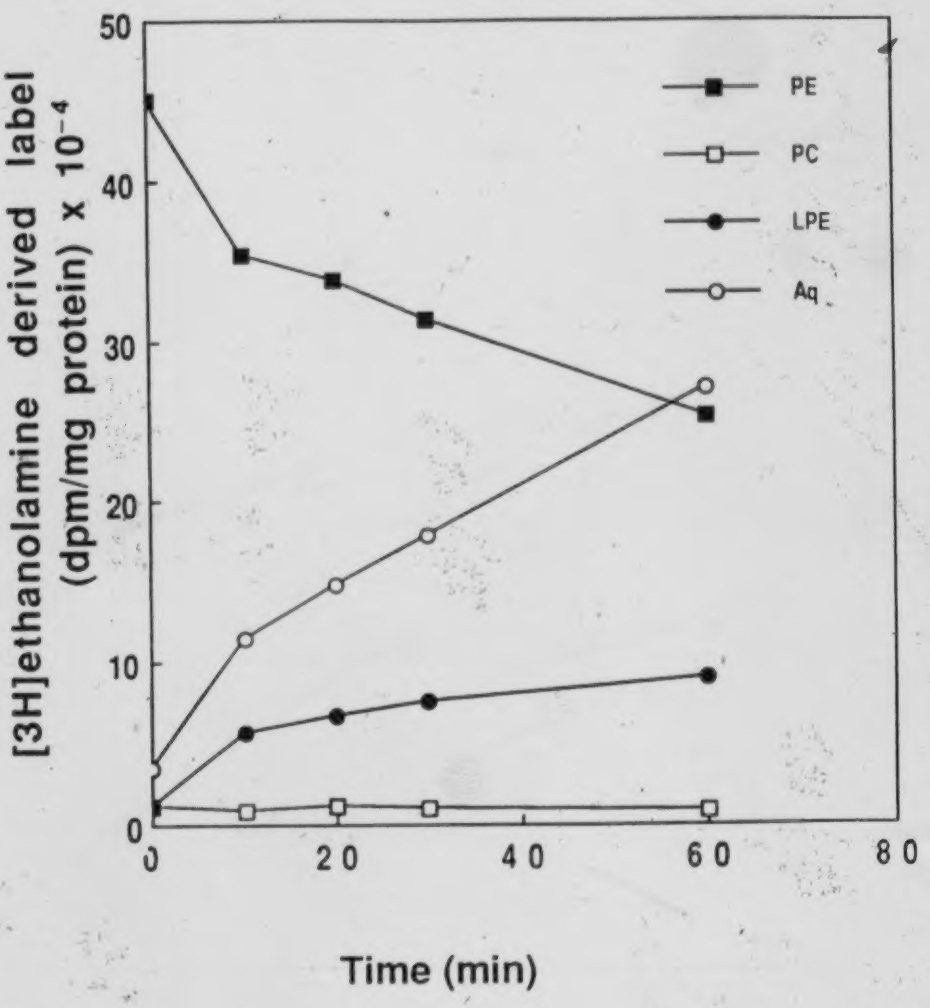
[3H]ethanolamine derived label
(dpm/mg protein) x 10^{-4}
50
40
30
20
10
0
0
20
40
60
80
Time (min)
PE
PC
LPE
Aq

FIGURE IV.2.

SPECIFIC RADIOACTIVITY OF MOLECULAR SPECIES OF MICROSOMAL PE DERIVED FROM [^{3}H]ETHANOLAMINE

Molecular species of PE, isolated from the experiments depicted in Fig. IV.1., were analyzed for radioactivity and phosphorus content as described under Materials and Methods. Values are the mean of four separate incubations ± S.E.M. The number on the x axis refer to the molecular species (7, 16:0-22:6; 8, 16:0-20:4; 9, 16:0-18:2; 10, 18:1-18:2; 11, 18:0-20:5 & 16:0-22:5; 13, 18:0-22:6; 14, 18:0-20:4; 15, 18:0-18:2; 16, 18:0-22:5).

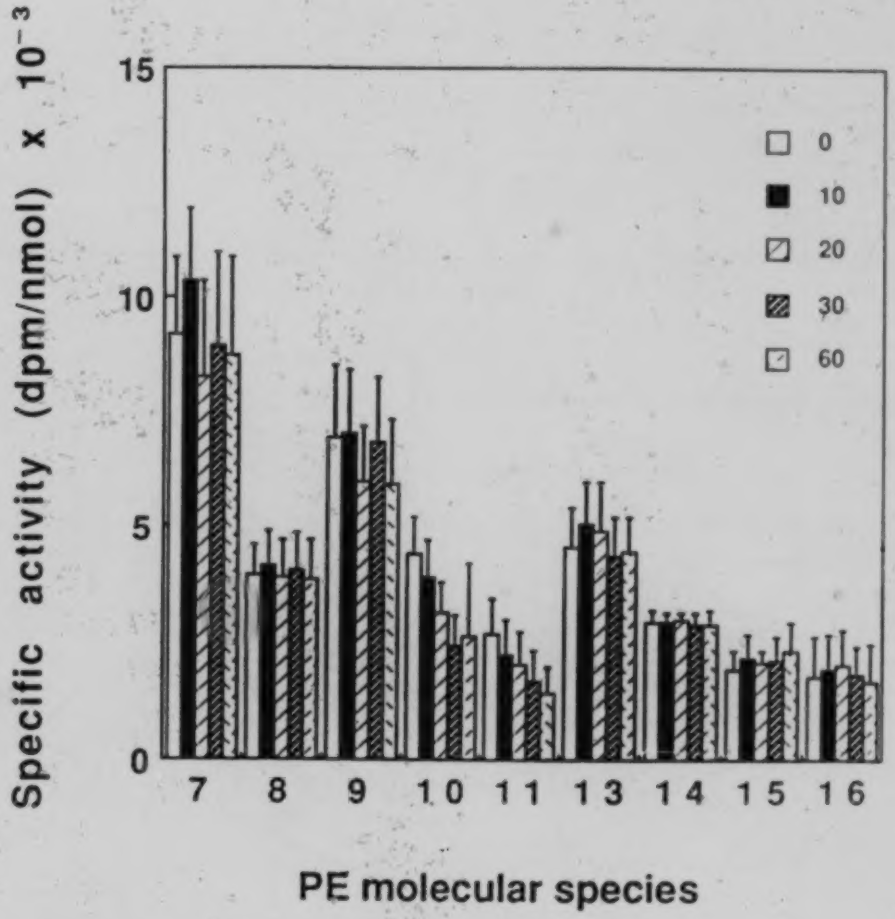
Specific activity (dpm/nmol) x 10^{-3}
15
10
5
0
☐ 0
■ 10
▨ 20
▨ 30
☐ 60
7
8
9
10
11
13
14
15
16
PE molecular species

FIGURE IV.3.

CATABOLISM OF MITOCHONDRIAL [^{3}H]ETHANOLAMINE-LABELED PE BY ENDOGENOUS PHOSPHOLIPASE ACTIVTY.

Mitochondrial fractions were prepared as described in the Materials and Methods. At the start of the experiment 200 µl of membranes were incubated with 1.0 ml of 50 mM Tris-HCl, pH 8.5 containing 10 mM $CaCl_2$. The samples were immediately placed in a 37°C shaking water bath (250 rpm) and incubated for the indicated times. The reaction was stopped by the addition of 4.8 ml of chloroform/methanol (2:1, v/v). The PC, PE and lysoPE were isolated by TLC and the amount of radioactivity was determined as described in the Materials and Methods. The amount of radioactivity was determined on an aliquot of the aqueous phase (Aq). The results are of a representative experiment.

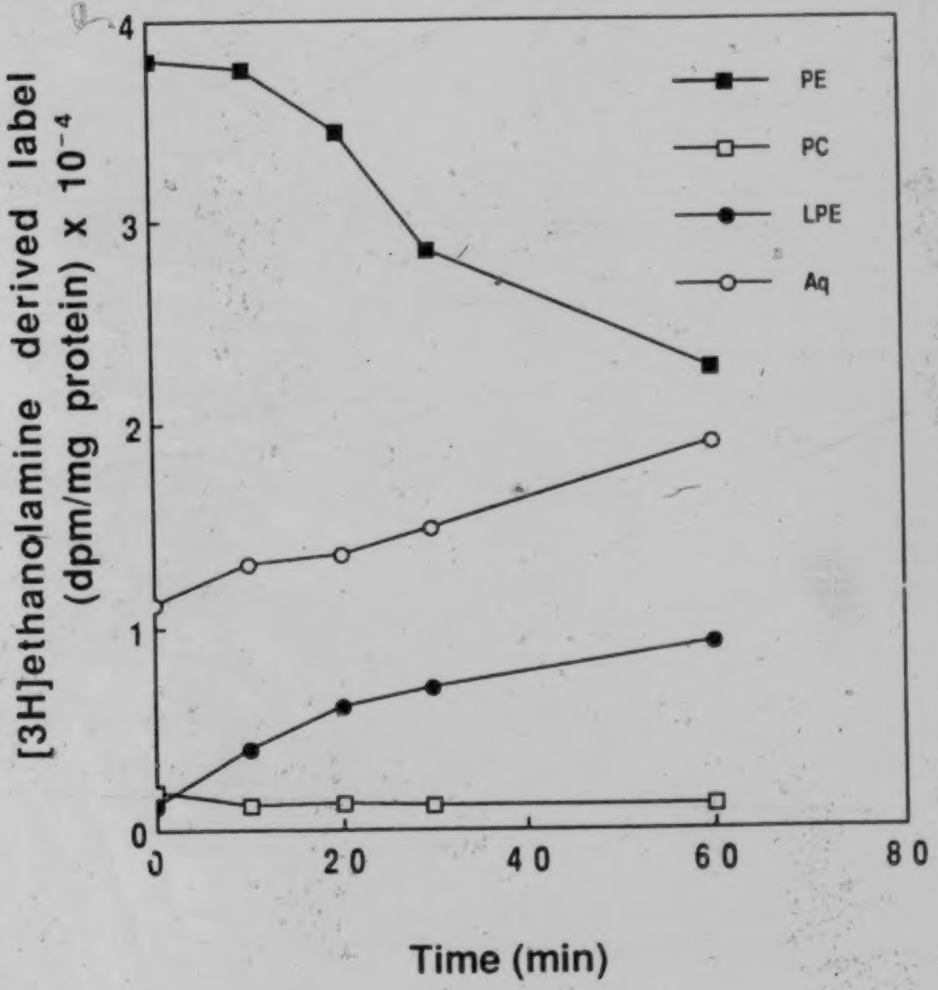
[3H]ethanolamine derived label (dpm/mg protein) x 10⁻⁴
0
1
2
3
4
0
20
40
60
80
Time (min)
PE
PC
LPE
Aq

FIGURE IV.4.

SPECIFIC RADIOACTIVITY OF MOLECULAR SPECIES OF MITOCHONDRIAL PE DERIVED FROM [^{3}H]ETHANOLAMINE

Molecular species of PE, isolated from the experiments depicted in Fig. IV.4., were analyzed for radioactivity and phosphorus content as described under Materials and Methods. Values are the mean of two separate incubations ± S.D. The number on the x axis refer to the molecular species (7, 16:0-22:6; 8, 16:0-20:4; 9, 16:0-18:2; 10, 18:1-18:2; 11, 18:0-20:5 & 16:0-22:5; 13, 18:0-22:6; 14, 18:0-20:4; 15, 18:0-18:2; 16, 18:0-22:5).

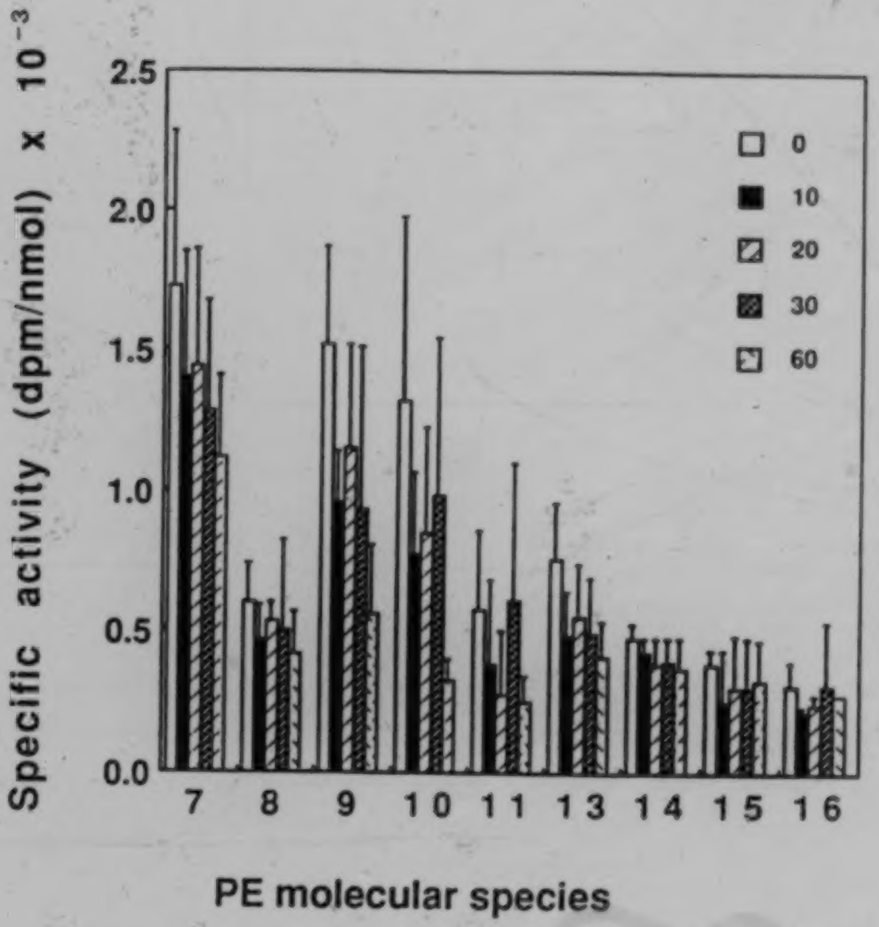
Specific activity (dpm/nmol) x 10^{-3}
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
60
7
8
9
10
11
13
14
15
16
PE molecular species

TABLE IV.1.

CHARACTERIZATION OF [^{3}H]ETHANOLAMINE-LABELED MICROSOMAL AND MITOCHONDRIAL FRACTIONS

500 µCi of [^{3}H]ethanolamine was injected into a female rat and after 10 min. the liver was removed and subcellular fractions were isolated as described in the Materials and Methods. The mitochondrial fraction was resuspended in 3 ml of buffer A and 200 µl aliquots (containing 5.9 mg protein) were stored at -70°C. The microsomes were re-solubilized in approximately 5 ml of buffer A and 200 µl aliquots (containing 5.2 mg protein) were stored at -70°C. The membrane protein concentration and the specific activities of PE and PC were determined as described in chapter III. The purity of these membrane preparations has been described previously (Vance, 1991). The results are the average ± mean of 4 determinations on one preparation.

Cell fraction	Mass (nmoles Pi/mg protein)	Specific activity (dpm/nmol)
Microsomes		
PE	90±2	4432±410
PC	230±7	67±35
Mitochondria		
PE	87±7	789±130
PC	91±3	23±6

TABLE IV.2.

PERCENT DISTRIBUTION OF [^{3}H]ETHANOLAMINE-LABELED PE MOLECULAR SPECIES IN MICROSOMES AS A FUNCTION OF TIME

Microsomes were prepared as described in the Materials and Methods. At the start of the experiment 200 µl of membranes were incubated with 1.0 ml of 50 mM Tris-HCl, pH 8.5 containing 10 mM $CaCl_2$. The samples were immediately placed in a 37°C shaking water bath (250 rpm) and incubated for the indicated times. The reaction was stopped by the addition of 4.8 ml of chloroform/methanol (2:1, v/v). The PE was isolated by TLC, eluted from the silica gel, and stored at -20°C prior to analysis by HPLC. Molecular species of PE were analyzed for radioactivity as described under Materials and Methods. The results are the mean ± SD of four determinations.

Molecular Species	mole % PE (T= 0 min)	% distribution [^{3}H]ethanolamine-label					mol% PE (T = 60 min)
		Time (min)					
		0	10	20	30	60	
16:0-22:6 (7)	12.5±0.6	28.5±4.3	27.2±1.0	27.7±2.7	25.3±2.8	24.6±4.9	9.3±1.2
16:0-20:4 (8)	8.4±0.3	6.7±0.4	7.1±0.3	7.5±0.8	7.2±1.4	7.2±1.2	7.5±1.4
16:0-18:2 (9)	5.5±0.6	11.1±1.9	10.1±1.8	11.9±0.4	10.7±2.5	8.7±0.6	7.0±1.3
18:1-18:2 (10)	3.5±0.5	5.7±0.5	5.9±0.3	6.7±1.4	6.2±1.3	5.0±2.1	6.3±1.6
18:0-20:5, 16:0-22:5 (11)	3.5±0.8	2.4±0.1	2.7±0.8	2.3±0.3	3.4±0.1	3.5±0.4	6.0±0.9
18:0-22:6 (13)	9.5±0.4	9.6±0.7	7.8±0.6	9.2±0.8	7.7±0.3	8.5±0.1	8.7±0.1
18:0-20:4 (14)	33.9±5.7	21.3±2.2	23.8±0.6	18.2±2.4	21.8±5.1	22.2±7.5	25.3±6.0
18:0-18:2 (15)	4.8±0.4	2.5±0.6	2.4±1.5	3.6±0.7	2.9±0.8	4.6±0.5	6.3±1.2
18:0-22:5 (16)	3.5±0.5	1.4±0.2	1.8±0.9	2.1±1.7	2.4±0.6	3.0±0.5	4.6±0.5

TABLE IV.3.

PERCENT DISTRIBUTION OF [^{3}H]ETHANOLAMINE-LABELED PE MOLECULAR SPECIES IN MITOCHONDRIA AS A FUNCTION OF TIME

Mitochondrial fractions were prepared as described in the Materials and Methods. At the start of the experiment 200 µl of membranes were incubated with 1.0 ml of 50 mM Tris-HCl, pH 8.5 containing 10 mM $CaCl_2$. The samples were immediately placed in a 37°C shaking water bath (250 rpm) and incubated for the indicated times. The reaction was stopped by the addition of 4.8 ml of chloroform/methanol (2:1, v/v). The PE was isolated by TLC, eluted from the silica gel, and stored at -20°C prior to analysis by HPLC. Molecular species of PE were analyzed for radioactivity as described under Materials and Methods. The results are the mean ± SD of two determinations.

Molecular Species	mol % PE (T= 0 min)	% distribution [^{3}H]ethanolamine-label					mol% PE (T= 60 min)
		Time (min)					
		0	10	20	30	60	
16:0-22:6 (7)	11.3±0.8	24.8±1.7	24.8±2.3	23.2±2.2	21.9±1.9	18.7±1.0	8.0±1.4
16:0-20:4 (8)	8.2±1.0	8.0±0.7	8.0±0.6	8.3±0.6	8.9±1.5	8.2±0.4	7.5±0.8
16:0-18:2 (9)	6.2±0.2	11.0±1.2	11.9±3.1	11.2±3.6	13.1±2.3	11.6±2.5	6.8±1.7
18:1-18:2 (10)	3.5±0.7	5.1±0.4	4.0±1.8	3.8±1.6	4.2±2.5	3.5±1.6	6.5±2.5
18:0-20:5, 16:0-22:5 (11)	3.5±0.9	2.1±0.4	2.1±0.3	2.0±0.3	1.8±0.6	1.9±0.7	4.2±1.5
18:0-22:6 (13)	9.3±0.7	10.2±0.7	11.6±0.5	12.3±1.2	12.3±2.1	11.9±2.3	8.3±0.7
18:0-20:4 (14)	30.8±8.3	24.3±3.2	24.1±1.6	25.8±0.8	27.7±4.5	26.7±2.0	27.3±10.4
18:0-18:2 (15)	7.0±1.4	3.4±0.8	3.6±1.1	3.1±0.9	4.0±0.8	4.0±1.2	6.2±1.8
18:0-22:5 (16)	3.4±1.6	0.9±0.2	1.6±1.2	1.8±1.2	1.6±0.7	1.8±1.3	3.9±1.2

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CHAPTER V.

METABOLISM OF PC IN CHOLINE DEFICIENT RAT HEPATOCYTES[1]

INTRODUCTION

Recently, we reported that, in isolated hepatocytes, PC synthesized from [^{3}H]choline was subject to extensive remodeling during a 24 h chase period (Samborski et al., 1990). However, the specific activities of all molecular species increased during the chase period (probably because the phosphocholine pool was highly labeled) and this complicated the interpretation of the remodeling studies. In this study molecular species of PC were separated by high-performance liquid chromatography (HPLC).

Choline-deficient hepatocytes have proven to be an excellent model to study the regulation of PC biosynthesis (Jamil et al., 1990), lipoprotein secretion (Yao and Vance, 1988) and recently, PC catabolism (Tijburg et al. 1991). In choline-deficient hepatocytes, PC can be rapidly and highly labeled from [^{3}H]choline without extensive accumulation of label in the phosphocholine pool (Tijburg et al. 1991). Therefore, it is possible to study remodeling of PC without the complication of continued biosynthesis of PC from prelabeled phosphocholine.

Recently we showed that supplementation of choline-deficient hepatocytes with choline increased PC catabolism, probably as the result of an increase in the concentration of PC (Tijburg et al. 1991). We now report extensive remodeling of PC in choline-deficient hepatocytes which involved both the *sn*-1 and the *sn*-2 position and which decreased upon choline supplementation of the medium.

[1] A version of this chapter has been published: Tijburg, L. B. M., Samborski, R.W., and Vance D.E. (1991) Biochim. Biophys. Acta (In press).

MATERIALS AND METHODS

Materials

Same as in chapter III with the addition that choline-deficient diet was obtained from ICN Biochemicals, Canada.

Animals and Hepatoctes

For these studies male Sprague-Dawley rats (45-50 g) were feed a choline-deficient diet for 3 days. The composition of the diet has previoulsy been described (Yao and Vance, 1988). Hepatocytes were isolated as decribed in Chapter II and the cells were plated on collagen-coated dishes at a density of 3 x 10^6 cells/dish in 3 ml Dulbecco's MEM containing 17% delipidated fetal calf serum (Cham and Knowles, 1976).

Pulse chase experiments

Hepatocytes were incubated for 4-5 h prior to the experiment. The dishes were washed once with 2 ml serum-free medium to remove serum and non-viable cells and further incubated for 30 min with 2 ml serum-free medium containing 10 µCi/dish [*methyl*-^{3}H]choline (15 Ci/mmol). The cells were washed twice with 2 ml choline- and methionine-free medium and subsequently, incubated for up to 12 h in 2 ml of the same medium in the absence or presence of 100 µM choline plus 100 µM methionine.

Experimental analysis

Same as described in Chapter III.

Subcellular fractionation

Male Sprague-Dawley rats were fed a choline-deficient diet for 4 days. The liver was perfused with phosphate-buffered saline and subsequently homogenized in 4 vols. of buffer containing 0.25 M sucrose, 20 mM Tris-HCl (pH=7.4) and 1 mM EDTA. Homogenates were centrifuged for 5 min at 600 x g. The supernatant was recentrifuged for 5 min at 600 x g. The supernatant of the latter step was centrifuged for 15 min at 10,000 x g. The 10,000 x g pellet was considered as the mitochondrial fraction. The

10,000 x g supernatant was centrifuged for 60 min at 100,000 x g resulting in a microsomal pellet. Microsomes and mitochondria were extracted (Folch, 1957) and after separation by TLC, PC was analyzed by HPLC as described in Chapter II..

Statistical analysis.

Results are presented as either the means ± S.E.M. or the means ± S.D. of the number of experiments indicated in the legends. Statistical analysis was done by student's *t*-test.

RESULTS

Hepatic PC was isolated from rats fed a choline-deficient or a choline-supplemented diet for 4 days and the composition of the molecular species was analyzed by HPLC (Table V.1.). As has been reported previously, 18:0-20:4 PC was the major species of hepatic PC (Trewhella and Collins, 1973; Ridgway and Vance, 1988; Pind et al., 1985). The amount of 18:0-20:4 PC in microsomes was slightly lower than observed by others (Trewhella and Collins, Pind et al., 1985). It has been shown previously that the phospholipid composition of membranes from livers from choline-deficient rats changed primarily with an increase in phosphatidylethanolamine and a decrease in PC (Lyman et al., 1973). However, the data in Table V.1. demonstrate that this decrease in PC was not accompanied by major changes in the distribution of the molecular species of PC in microsomes or mitochondria. The content of 16:0-20:4 PC in microsomes from choline-deficient liver was slightly lower than in livers from control rats ($P<0.025$, n=3). In mitochondria from choline-deficient livers the relative amount of 16:0-18:1 and 16:0-18:2 PC was slightly lower than in mitochondria from choline-supplemented livers, whereas the amount of 18:0-20:4 PC was slightly enhanced (Table V.1.).

Cultured rat hepatocytes, isolated from a choline-deficient rat, were incubated for 30 min with [methyl-^{3}H]choline and chased for up to 12 h in choline- and methionine-free medium (CD) or in medium containing 100 µM choline and 100 µM methionine (CS). At the indicated times PC mass and radioactivity were determined. PC was fractionated into molecular species by HPLC. At the end of the chase period the distribution of the radioactivity of most species from CD cells approximated the mol % distribution (Table V.2.).

Analysis of the molecular species of PC showed that at the end of the pulse 75% of the label was incorporated into palmitate-containing species and 16.5% in stearate-containing species (Table V.2.). During the 0 to 12 h chase period radiolabel decayed from 16:0-22:6, 16:0-20:4, 16:0-18:2 and 16:0-18:1, and 18:0-22:6 (8.95 x 10^5, 2.43 x 10^5, 15.48 x 10^5, 28.91 x 10^5 and 1.86 x 10^5 dpm, respectively). During this same period there was a concomitant increase in 18:0-20:4, 18:0-18:2, and 18:0-18:1 PC (9.64 x 10^5, 3.29 x 10^5, and 0.81 x 10^5 dpm, respectively). After 12 h, 56% of the total radioactivity was associated with palmitate-containing species, whereas 37% was recovered in stearate-containing species.

Recently, Samborski et al. (1990) demonstrated that expression of the data as percentage of the total radioactivity may lead to incorrect conclusions. Therefore, we calculated the specific activities of the major molecular species of PC from choline-deficient hepatocytes. Fig. V.1. clearly indicates a precursor-product relationship between palmitate- and stearate-containing species. Initially, the highest specific activity was observed in 16:0-18:1 (peak 12) and 16:0-18:2 PC (peak 9). After 12 h the specific activity of 16:0-18:2 PC was decreased to 52% of the initial value, whereas the specific activity of 16:0-18:1 decreased by approximately 40%. Concomitantly, the specific activity of 18:0-20:4 species (peak 14) increased by 2.4-fold and that of 18:0-18:2 (peak 15) by 3.4-fold. Apparently, 16:0-18:1 and 16:0-18:2 PC were extensively converted to 18:0-20:4 and 18:0-18:2 species (Fig. V.1.).

Supplementation of choline-deficient hepatocytes with choline leads to a significant increase in cellular PC during the first 4 h of the choline treatment (Yao and Vance, 1988; Tijburg, 1991). Despite the abundance of newly synthesized PC in choline-supplemented cells, the decrease in percentage of the radioactivity in 16:0-18:2 (peak 9) and 16:0-18:1 PC (peak 12) was similar to choline-deficient cells (cf Tables V.2. and V.3.). However, the percent increase in radioactivity associated with 18:0-20:4 PC (peak 14) was much less pronounced in choline-supplemented cells (+6.3%) than in choline-deficient cells (+13.2%).

From measurement of the masses of the major molecular species in choline-supplemented hepatocytes, the specific activity could be calculated (Fig. V.2.). In every case, except 18:0-18:2 (peak 15) and 18:0-20:4 (peak 14), the specific activity decreased during the initial 2 h incubation in the presence of choline. The data in Table V.3. and Fig. V.2. are consistent with deacylation-reacylation of some molecular species in choline-supplemented cells, although the transformation can not yet be proven.

Taken together, the results indicate that in choline-deficient as well as choline-supplemented cells there is remodeling of 16:0-18:1 and 16:0-18:2 to 18:0-18:2 and 18:0-20:4 PC. The increase in stearate-containing species is more pronounced in choline-deficient cells than in choline-supplemented cells.

DISCUSSION

Remodeling of de novo synthesized PC has been studied in liver (reviewed in Holub and Kuksis, 1978) and, more recently, extensively in lung (reviewed van Golde et al., 1988). In most studies argentation thin-layer chromatography was used to separate classes of phospholipids with different degrees of unsaturation. Recent advances in analytical techniques made it possible to resolve molecular species of phospholipids by HPLC (Patton et al., 1982).

Analysis of molecular species of hepatic PC isolated from rats fed a choline-deficient or choline-supplemented diet (Table V.1.) showed that the amount of 18:0-20:4 PC was slightly lower than the value reported by others (Trewhella and Collins, 1973; Pind et al., 1985). However, the choline-deficient diet used in our experiments contained a relatively high amount of sucrose (56%) compared to the regular chow diet (Yao and Vance, 1988). Groener et al. (1979) reported that the amounts of stearate and arachidonate associated with PC were decreased in livers from fasted rats fed a high-sucrose, fat-free diet. Thus, the lower amount of 18:0-20:4 PC might be explained by the high sucrose content of the diet.

Comparison of the distribution of the molecular species of PC from choline-deficient and choline-supplemented livers showed that choline deficiency did not significantly change the composition of PC species (Table V.1.). Our observations are in agreement with those of Lyman et al. (1973) who reported that the fatty acid composition of PC was relatively unaffected by choline deficiency. This indicates that, despite a reduction of PC synthesis by 70% (Yao and Vance, 1988) and significant alterations in the phospholipid compositon of the cell (Lyman et al., 1973), the distribution of molecular species of PC was maintained during choline deficiency.

It has been shown previously that biosynthesis of PC via the CDP-choline pathway was primarily involved in the formation of monoenoic and dienoic species (Arvidson, 1968; Åkesson et al., 1970; Trewhella and Collins, 1973; Vereyken et al, 1972; Sundler et al., 1972; Kanoh, 1969). The present data demonstrate that there was a preferential incorporation of labeled choline into 16:0-18:1 (peak 12) and 16:0-18:2 (peak 9) PC (Table V.2.). At the beginning of the chase period, the specific activity of each of the two molecular species was at least 2-fold higher than that of any of the other species (Figs. V.1 and V.2). Subsequent incubation of the cells in choline- and methionine-free medium resulted in a decrease of the specific activity of 16:0-18:1 (peak 12) and 16:0-18:2 (peak 9) molecular species (Fig. V.1.). This effect was accompanied by an increase of the specific activity of 18:0-20:4 (peak 14) and 18:0-18:2 (peak 15) PC. This indicates that there was a precursor-product relationship between 16:0-18:1 and 16:0-18:2 PC on the one hand and 18:0-18:2 and 18:0-20:4 PC on the other. There is good agreement between our data and earlier reports that there is a preferential incorporation of palmitate over stearate in *de novo* synthesized PC (Arvidson, 1968; Elovson, 1965; Kuksis et al. 1975a; Kuksis et al. 1975b). Holub (1978) observed that cholinephosphotransferase, the last enzyme of the CDP-choline pathway, showed a marked preference for 1-palmitoyl species over 1-stearoyl species of diacylglycerol. Thus, there is preferential formation of PC species containing palmitate in the *sn*-1 position.

While it is accepted that remodeling of phospholipids can occur at both the *sn*-1 and *sn*-2 position (Holub, 1980; Thompson and Belina, 1986) it is generally assumed that remodeling of PC mainly involves *sn*-2 remodeling. The precursor-product relation between 16:0-18:2 (peak 9) and 16:0-18:1 (peak 12) PC and 18:0-20:4 (peak 14) species as observed in Fig. 1, implies remodeling of PC at both the *sn*-1 and the *sn*-2 position. This conversion may begin with deacylation of palmitate-containing species by phospholipase A_1, followed by reacylation of the 2-acylglycerophosphocholine. Åkesson et al. (1976) provided *in vivo* evidence to support the preferential reacylation of 2-linoleoyl-sn-glycerophosphocholine with stearic acid. The stearate may be incorporated into the lysoPC molecule via an acyl-CoA:2-acylglycerophosphocholine acyltransferase. Alternatively, stearate may be incorporated into the *sn*-1 position via the action of CoA-dependent transacylase system (transfer of an acyl residue from another phospholipid) present in liver microsomes (Sugiure et al., 1988). The exact contribution that these two pathways makes towards stearate incorporation is unknown. Several studies have indicated that arachidonic acid enters rat liver PC mainly by acylation of 1-acyl-*sn*-glycerophosphocholine (Kanoh, 1969; Holub and Kuksis, 1971; Kanoh and Ohno, 1975). An acyltransferase showing selectively towards 20:4-CoA has been partially purified from rat liver microsomes (Hasagawa-Saaki and Ohno, 1980). However, remodeling of the *sn*-2 position initially requires the action of a phospholipase A_2. Since Ca^{2+}-dependent, alkaline pH optimum phospholipase A_2 is predominantly located in the mitochondria (Waite, 1987; Aarsman et al., 1989) the *sn*-2 remodeling of newly-made PC would require either transport to the mitochondria or the presence of a phospholipase A_2 activity in microsomes. However, Inada et al. (1991) have recently raised questions concerning the cellular origin of the rat liver mitochondria PLA_2. Using a polyclonal antibody raised against PLA_2 from rat spleen membrane, they demonstrated that rat liver Kupffer cells contain 6-fold greater activity than hepatocytes. Recent work by Wilton (1991) demonstrated that the rat liver mitochondria PLA_2 is not actually present in the hepatocyte at all. Wilton has suggested that the "mitochondrial PLA_2" is actually derived from Kupffer cells and becomes associated with the mitochondrial fraction during isolation of the organelle. Consistant with this hypothesis is the observation that the first 24 amino acids of the rat liver PLA_2 (Aarsman et al., 1989) showed 96% identity with that of rat platelet (Hayakawa et al., 1987) and rat spleen membrane-associated PLA_2 (Ono et al. , 1988). While these results strongly suggest alternative sources for the rat liver PLA2 they do not suggest that the mitochondria or the hepatocyte lacks PLA_2 activity. Thus, the mitochondria may yet contain a PLA_2 which is involved in the deacylation-reacylation pathway. Furthermore, since microsomes contain phospholipase A_1 activity and no phospholipase A_2 activity (Waite, 1987) it would appear that the mitochondria may be involved in remodeling of newly-made PC. In summary, the biosynthesis of much of 18:0-20:4 PC appears to involve a multi-step remodeling reaction of 16:0-18:1 or 16:0-18:2 PC which takes place in both the endoplasmic reticulum (ER) and the mitochondria.

We present an hypothetical model for this transformation in Fig. V.3. 16:0-18:1 PC is mainly synthesized via the CDP-choline pathway on the endoplasmic reticulum. In the first remodeling step palmitate would be removed by a phospholipase A_1 (step 2). Subsequently, the 2-acyl-lysoPC would be reacylated with stearate (step 3). The donor for this may be either stearoyl-CoA or 1-stearoyl-2 acyl glycerophospholipid. Since the mitochondrial phospholipase A_2 is implicated in the deacylation of the *sn*-2 fatty acid and the composition of mitochondria and microsomes do not differ significantly the formation of 18:0-20:4 PC should include a reversible transport mechanism between the ER and mitochondria (step 4). Finally, oleate would be removed from the *sn*-2 position by phospholipase A_2 and be replaced by arachidonate, the donor most probably being 20:4-CoA (Fig. 4, steps 5 and 6). We recognize that this model is probably oversimplified and that Ca^{2+}-dependent and -independent phospholipases in the cytosol and lysosomes may be actively involved. Also, while much of the current evidence implicates the microsomal PLA_1 and the mitochondrial PLA_2 in these reactions, the exact location of these enzyme activies is unkown. In light of these short comings, we present this working model in hope that it will be useful in elucidating the exact pathway by which PC species are remodeled.

Recently, our lab has demonstrated that in cells prelabeled with [^{3}H]choline, addition of choline to the chase medium resulted in an increase in the degradation of labeled PC with a concomitant increase of the formation of [^{3}H]glycerophosphocholine (Tijburg et al. 1991). It was proposed that this effect was due to an increase of the cellular concentration of PC (Tijburg et al. 1991). Comparison of the data in Tables V.2. and V.3. demonstrates that remodeling of PC was more extensive in choline-deficient cells than in choline-supplemented cells. Although the decrease in radioactivity in 16:0-18:1 (peak 12) and 16:0-18:2 PC (peak 9) in choline-supplemented was only slightly lower than in choline-deficient cells, choline supplementation significantly reduced the increase of the radioactivity associated with 18:0-20:4 PC (peak 14) as observed in choline-deficient cells. This suggests that reacylation of lysoPC in choline-supplemented cells was lower than in choline-deficient cells. These findings are further supported by the analysis of the data in Fig. V.2.. During the first 2 h of the chase the specific activity of peaks 9 and 12 decreased by 48% and 41% respectively. These changes are only partially accounted for by an increase in the concentration of cellular PC due to supplementation with choline and appear also to involve deacylation reactions. During the same period there was not a concomitant increase in the specific activity of stearate-containing species (Fig. V.2., peaks 14,15). Thus the relative rates of the deacylation reactions in choline-supplemented cells were increased with respect to the reacylation reactions compared to choline-deficient cells. These results agree with the previous demonstration that the formation of glycerophosphocholine was enhanced in choline-supplemented cells (Tijburg et al. 1991). The increased catabolism of PC in choline-supplemented cells as observed previously (Tijburg et al. 1991), might be explained by a relative increase of the deacylation of lysoPC as compared to reacylation of this phospholipid. Apparently, the regulation of deacylation and reacylation of lysoPC plays an important role in the regulation of PC concentration in the cell.

FIGURE V.1.

SPECIFIC RADIOACTIVITY OF MOLECULAR SPECIES OF [^{3}H]CHOLINE-LABELED PC ISOLATED FROM CD HEPATOCTES

CD hepatocytes were pulsed for 30 min with 10 µCi/dish [*methyl*-^{3}H]choline. The pulse medium was removed, the monolayers washed and fresh choline- and methionine-free medium was added. At the indicated times the cells were harvested and PC was isolated. After further fractionation by HPLC the amount of radioactivity and lipid phosphorus associated with each peak was determined. Data are the mean ± S. E. of four separate experiments. The numbers on the x axis refer to the HPLC peak corresponding to the molecular species (7, 16:0-22-6; 8, 16:0-20:4; 9, 16:0-18:2; 12, 16:0-18:1; 13, 18:0-22-6; 14, 18:0-20:4; 15, 18:0-18:2).

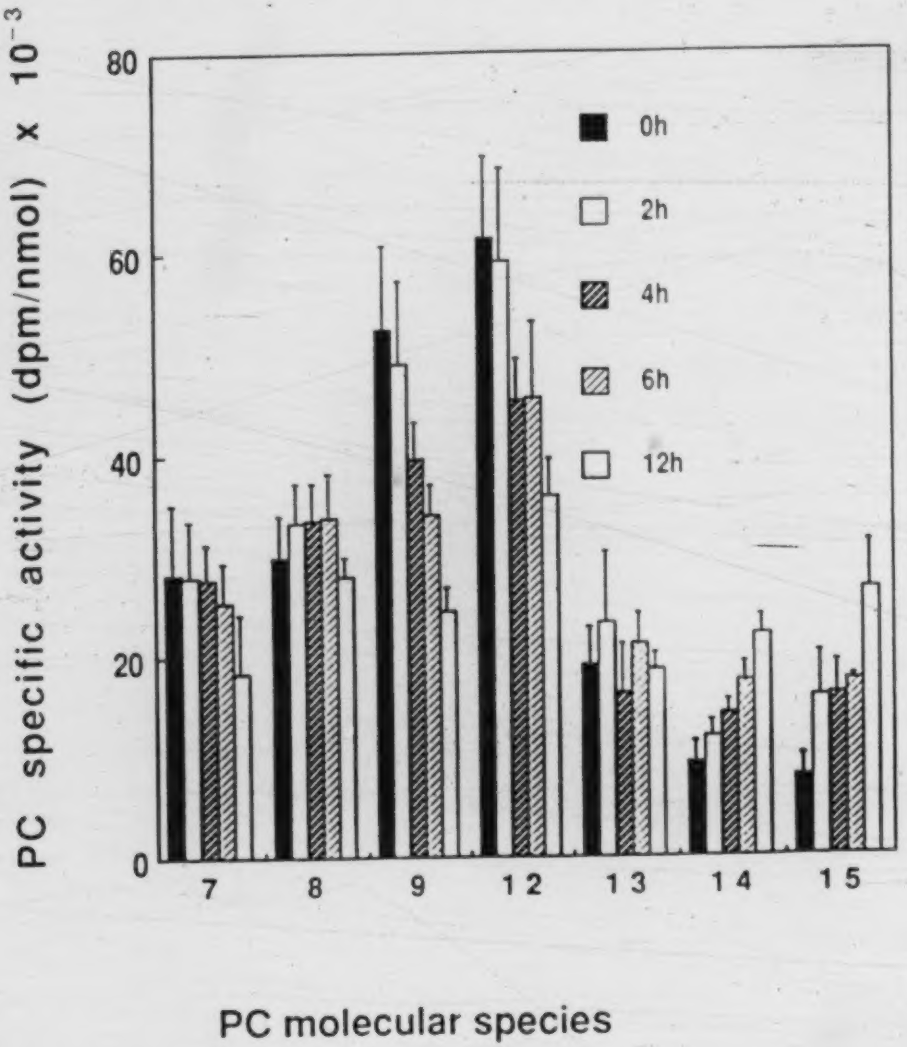
PC specific activity (dpm/nmol) x 10^{-3}
0
20
40
60
80
0h
2h
4h
6h
12h
7
8
9
12
13
14
15
PC molecular species

FIGURE V.2.

SPECIFIC RADIOACTIVITY OF MOLECULAR SPECIES OF [^{3}H]CHOLINE-LABELED PC ISOLATED FROM CS HEPATOCTES

CD hepatocytes were pulsed for 30 min with 10 µCi/dish [*methyl*-^{3}H]choline. The pulse medium was removed, the monolayers washed and fresh medium containing 100 µM choline and 100 µM methionine was added. At the indicated times the cells were harvested and PC was isolated. After further fractionation by HPLC the amount of radioactivity and lipid phosphorus associated with each peak was determined. Data are the mean ± S. E. of four separate experiments. The numbers on the x axis refer to the HPLC peak corresponding to the molecular species (7, 16:0-22-6; 8, 16:0-20:4; 9, 16:0-18:2; 12, 16:0-18:1; 13, 18:0-22-6; 14, 18:0-20:4; 15, 18:0-18:2).

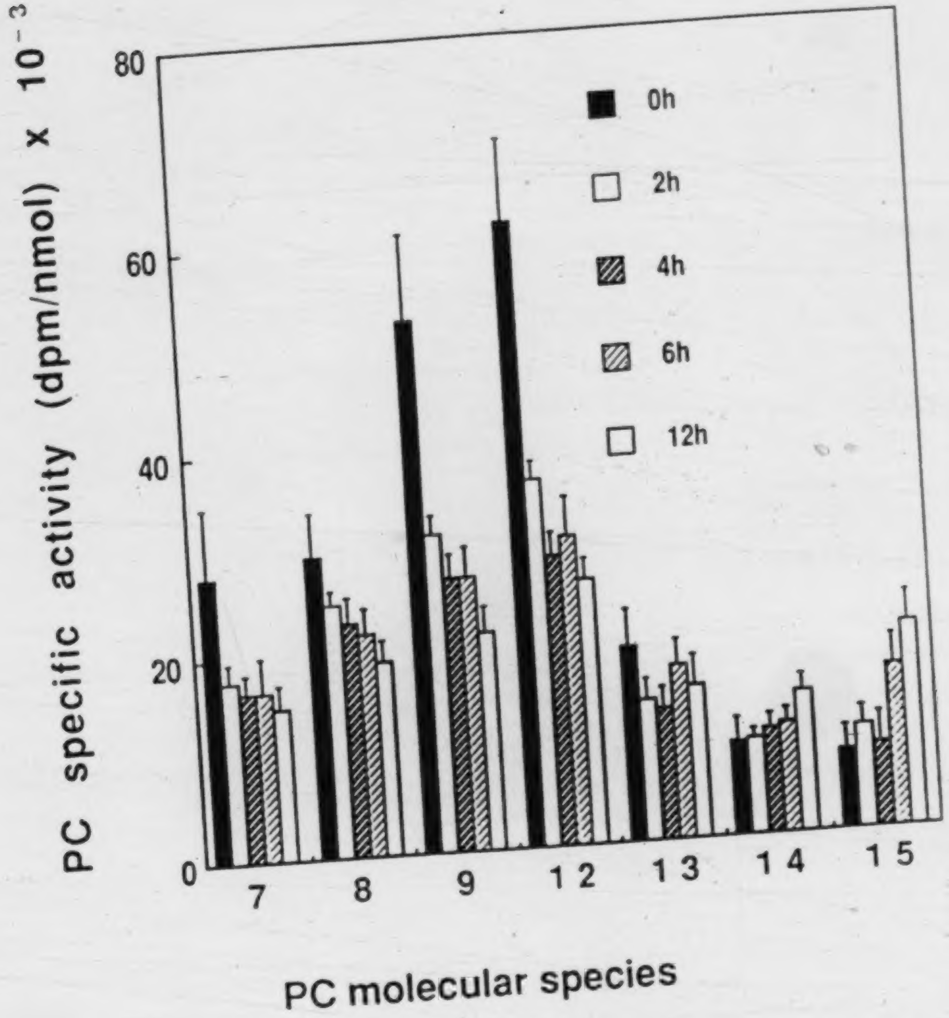
PC specific activity (dpm/nmol) x 10⁻³
80
60
40
20
0
0h
2h
4h
6h
12h
7
8
9
12
13
14
15
PC molecular species

FIGURE V.3.

HYPOTHETICAL MODEL FOR THE TRANSFORMATION OF 16:0-18:1 PC TO 18:0-20:4 PC

1, Biosynthesis of PC via the CDP-choline pathway; 2, deacylation of 16:0-18:1-PC by phospholipase A_1; 3, reacylation of 2-acyl PC with stearate, derived from either stearoyl-CoA or stearate-containing phospholipids; 4, transport of 18:0-18:1-PC from the endoplasmic reticulum to the mitochondria; 5, removal of the oleic acid by phospholipase A_2; 6, reacylation of 1-acyl PC with arachidonyl-CoA; 7, reversible transport of 18:0-20:4 PC between endoplasmic reticulum and mitochondria; C, choline. Alternatively, this remodeling mechanism may commence with *sn*-2 remodeling occurring first.

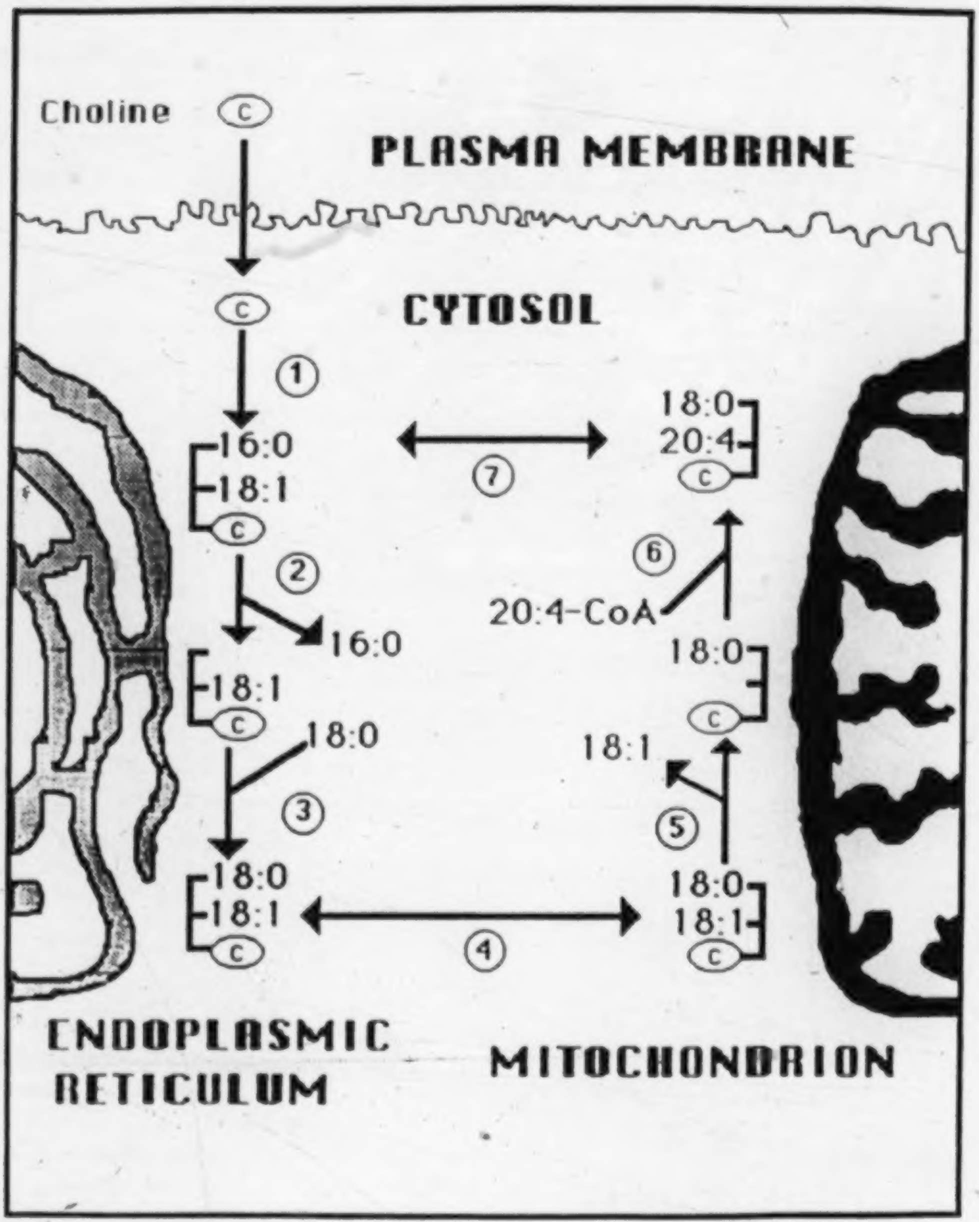
Choline
C
PLASMA MEMBRANE
C
CYTOSOL
①
16:0
18:1
C
⑦
18:0
20:4
C
②
16:0
⑥
20:4-CoA
18:1
C
18:0
18:0
C
③
18:1
⑤
18:0
18:1
C
④
18:0
18:1
C
ENDOPLASMIC RETICULUM
MITOCHONDRION

TABLE V.1.

CHARACTERIZATION OF PC SPECIES FROM THE LIVER OF CD AND CS RATS

Total lipid was extracted from the microsomal and mitochondrial fractions of rat liver and from monolayers of hepatocytes. Phospholipids were separated by TLC and PC was eluted from the gel. PC was further fractionated by HPLC and the lipid phosphorus was determined following perchloric acid digestion. Microsomes and mitochondria were isolated from the livers of CD and CS rats as described in Materials and Methods. Data are the mean ± S. D. of three animals. Hepatocytes were isolated from the livers of CD rats as described in Materials and Methods and then grown in the presence or absence of 100 μM choline plus 100 μM methionine for 12 h. Data are the means ± S. D. of four separate experiments.

peak #	molecular species	microsomes[a] CD	microsomes[a] CS	mitochondria CD	mitochondria CS	hepatocytes[b] CD	hepatocytes[b] CS
				mole % of total ± SD			
1,2	18:2-18:3, 14:0-22:6	1.8 ±0.4	1.2 ±0.2	1.0±0.1	0.9 ±0.6	0.7 ±0.5	0.7 ±0.7
3,4	16:1-18:2,18:2-22:6	4.9 ±1.1	4.6 ±1.8	1.9 ±0.3	1.3 ±0.4	1.0 ±0.8	1.1 ±1.0
5,6	18:2-20:4,18:2-18:2	3.0 ±2.5	5.3 ±1.6	2.9 ±0.4	2.3 ±0.4	1.1 ±0.8	0.9 ±0.6
7	16:0-22:6	6.3 ±0.3	5.8 ±1.0	6.6 ±1.0	6.3 ±1.0	7.5 ±2.2	12.9 ±3.2
8	16:0-20:4	11.4 ±0.6	13.7 ±1.2	14.1 ±1.9	14.4 ±0.6	20.9 ±3.0	19.7 ±3.1
9	16:0-18:2	8.5 ±0.2	8.1 ±0.4	7.9 ±0.1	9.8 ±1.4	8.4 ±1.5	8.9 ±0.3
10	18:1-18:2	4.7 ±0.2	3.3 ±0.2	3.5 ±0.2	3.9 ±0.5	2.3 ±0.3	2.7 ±0.4
11	18:0-20:5,16:0-22:5	5.4 ±0.6	5.3 ±0.5	5.3 ±0.6	5.2 ±0.7	5.0 ±3.4	2.9 ±0.8
12	16:0-18:1	11.6 ±0.4	10.8 ±1.3	10.5 ±0.4	13.5 ±2.3	12.5 ±1.5	16.0 ±2.0
13	18:0-22:6	6.6 ±0.5	6.6 ±1.5	6.4 ±0.2	6.6 ±0.6	7.4 ±1.4	8.8 ±1.4
14	18:0-20:4	19.1 ±1.4	21.2 ±1.7	24.4 ±0.1	21.6 ±2.1	23.2 ±4.0	19.0 ±3.5
15	18:0-18:2	6.2 ±0.8	6.9 ±1.5	6.0 ±0.2	6.9 ±0.7	4.8 ±2.0	2.6 ±1.4
16	18:0-22:5	2.7 ±0.6	2.3 ±1.2	2.3 ±1.0	1.8 ±0.4	1.5 ±0.4	1.3 ±0.7
17	18:0-18:1	3.9 ±0.1	4.9 ±1.6	4.0 ±0.2	3.0 ±1.3	2.8 ±0.6	1.7 ±0.7

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[a] Microsomes and mitochodria were isolated from the livers of CD and CS rat as described in Materials and Methods. Data are the mean ± SD of 3 animals.

[b] Hepatocytes were isolated from the livers of CD rats as described in Materials and Methods and then grown in the presence or absence of 100 uM choline plus 100 um methionine for 12 h. Data are the means ± SD of 4 separate experiments.

TABLE V.2.

METABOLISM OF [*METHYL*-^{3}H]CHOLINE-LABELED PC SPECIES IN CD HEPATOCYTES

CD hepatocytes were pulsed for 30 min with 10 µCi/dish [*methyl*-^{3}H]choline. The pulse medium was removed, the monolayers washed and fresh choline- and methionine-free medium was added. At the indicated times the cells were harvested and PC was isolated. After further fractionation by HPLC the amount of radioactivity and lipid phosphorus associated with each peak was determined. Data are the mean ± S. E. of four separate experiments.

peak #	molecular species	h 0	2	4	6	12	mole % cellular PC[a]
		% total dpm ± SD					
1,2	18:2-18:3, 14:0-22:6	1.4 ±0.7	1.2 ±0.4	1.2±0.7	0.9 ±0.2	0.5 ±0.1	1.3 ±0.9
3,4	16:1-18:2,18:2-22:6	1.7 ±1.0	1.7 ±0.5	1.4 ±0.3	0.9 ±0.4	1.0 ±0.2	2.0 ±1.9
5,6	18:2-20:4,18:2-18:2	1.8 ±0.9	1.5 ±0.7	1.4 ±0.4	1.3 ±0.3	1.1 ±0.1	1.1 ±1.0
7	16:0-22:6	9.1 ±2.2	8.2 ±2.1	7.8 ±2.7	7.5 ±2.4	4.7 ±2.9	16.6 ±2.1
8	16:0-20:4	17.4 ±1.8	21.1 ±1.0	21.2 ±4.7	21.9 ±1.3	23.5 ±1.6	8.9 ±1.0
9	16:0-18:2	16.6 ±2.3	14.6 ±1.9	12.0 ±2.8	13.1 ±4.0	9.4 ±2.2	2.9 ±0.1
10	18:1-18:2	3.6 ±0.2	2.7 ±0.5	2.4 ±0.6	2.2 ±0.5	1.8 ±0.3	3.3 ±1.4
11	18:0-20:5,16:0-22:5	2.0 ±0.4	2.3 ±0.6	2.3 ±0.7	2.4 ±0.5	3.1 ±0.7	3.2 ±1.4
12	16:0-18:1	31.3 ±1.2	28.4 ±1.7	24.4 +6.0	20.8 ±3.0	18.0 ±2.2	14.3 ±1.4
13	18:0-22:6	5.0 ±1.0	4.4 ±0.5	3.9 ±2.1	6.4 ±2.1	5.6 ±1.3	7.6 ±0.7
14	18:0-20:4	7.5 ±2.6	9.6 ±1.5	11.0 ±2.1	13.9 ±1.5	20.7 ±2.5	23.0 ±4.7
15	18:0-18:2	1.1 ±0.3	2.3 ±0.7	3.1 ±0.9	2.0 ±1.0	4.9 ±1.7	4.6 ±2.2
16	18:0-22:5	0.5 ±0.2	1.0 ±0.3	1.4 ±0.8	2.9 ±1.5	1.2 ±0.6	2.1 ±0.5
17	18:0-18:1	0.4 ±0.2	0.6 ±0.2	0.9 ±0.2	1.0 ±0.2	1.4 ±0.3	1.9 ±1.3

[a] The results at time = 0 h of the chase. The mole % distribution did not vary during the chase.

TABLE V.3.

METABOLISM OF [*METHYL*-^{3}H]CHOLINE-LABELED PC SPECIES IN CS HEPATOCYTES

CD hepatocytes were pulsed for 30 min with 10 µCi/dish [*methyl*-^{3}H]choline. The pulse medium was removed, the monolayers washed and fresh medium containing 100 µM choline and 100 µM methionine was added. At the indicated times the cells were harvested and PC was isolated. After further fractionation by HPLC the amount of radioactivity and lipid phosphorus associated with each peak was determined. Data are the mean ± S. E. of four separate experiments.

peak #	molecular species	h 0	 2	 4	 6	 12	mole% cellular PC[a]
		% total dpm ± SD					
1,2	18:2-18:3, 14:0-22:6	1.4 ±0.7	1.1 ±0.2	0.9 ±0.2	0.9 ±0.2	0.7 ±0.1	1.3 ±0.9
3,4	16:1-18:2,18:2-22:6	1.7 ±1.0	2.1 ±0.2	1.7 ±0.1	1.4 ±0.2	1.1 ±0.3	2.0 ±1.9
5,6	18:2-20:4,18:2-18:2	1.8 ±0.9	1.4 ±0.2	1.6 ±0.3	1.4 ±0.5	1.4 ±0.5	1.1 ±1.0
7	16:0-22:6	9.1 ±2.2	9.0 ±3.7	9.1 ±2.0	9.7 ±2.8	10.3 ±3.4	16.6 ±2.1
8	16:0-20:4	17.4 ±1.8	20.0 ±1.6	20.2 ±1.9	19.2 ±1.4	19.7 ±1.6	8.9 ±1.0
9	16:0-18:2	16.6 ±2.3	14.0 ±2.3	13.2 ±2.1	12.9 ±2.0	10.1 ±1.1	2.9 ±0.1
10	18:1-18:2	3.6 ±0.2	2.8 ±0.7	3.1 ±0.8	2.4 ±0.3	2.6 ±0.3	3.3 ±1.4
11	18:0-20:5,16:0-22:5	2.0 ±0.4	2.6 ±0.7	2.5 ±0.8	2.8 ±0.5	2.7 ±0.8	3.2 ±1.4
12	16:0-18:1	31.3 ±1.2	28.2 ±1.5	26.0 +2.9	23.7 ±4.2	22.3 ±3.5	14.3 ±1.4
13	18:0-22:6	5.0 ±1.0	4.9 ±1.0	4.8 ±1.0	8.4 ±2.2	6.8 ±0.8	7.6 ±0.7
14	18:0-20:4	7.5 ±2.6	8.7 ±1.0	10.3 ±1.8	10.4 ±1.8	13.8 ±2.6	23.0 ±4.7
15	18:0-18:2	1.1 ±0.3	2.1 ±0.4	1.8 ±1.0	3.1 ±0.6	3.1 ±1.0	4.6 ±2.2
16	18:0-22:5	0.5 ±0.2	0.5 ±0.1	1.3 ±0.8	0.7 ±0.2	0.7 ±0.4	2.1 ±0.5
17	18:0-18:1	0.4 ±0.2	0.5 ±0.1	0.7 ±0.2	0.9 ±0.3	1.1 ±0.4	1.9 ±1.3

a The results at time = 0 h of the chase. The mole % distribution did not vary during the chase.

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CHAPTER VI.

METABOLISM OF [^{3}H]SERINE-LABELED PE AND PC IN RAT HEPATOCYTES

INTRODUCTION

In the hepatocyte, there are at least three separate sites of phospholipid biosynthesis: 1) the endoplasmic reticulum; 2) the Golgi body; and 3) the mitochondria. The endoplasmic reticulum is the primary site for the synthesis of PC, by the CDP-choline and methylation pathways; PE, by the CDP-ethanolamine pathway; and PS, by the base-exchange pathway (Vance and Vance, 1988). These enzymatic systems are found to a lesser degree in the Golgi. The mitochondria lacks the above mentioned systems, but is unique in its ability to synthesize PE via PS decarboxylation (Dennis and Kennedy, 1972). These separate sites of biosynthesis have the potential of creating multiple pools of phospholipid within the hepatocyte. It has earlier been suggested that at least four specific pools of PC may exist in the hepatocyte, one each derived from choline and ethanolamine and two derived from serine (Vance and Vance, 1986a). These authors demonstrated that specific pools of phospholipids are used for lipoprotein biosynthesis. Multiple pools may also be created within the cell by the selective transfer or targeting of newly made phospholipids to intracellular membranes. While the intracellular phospholipid transfer proteins have been implicated in this process there is no direct evidence to support their involvement. Clearly, the initial site of biosynthesis plays a major role in determining the intracellular fate of the phospholipid.

In the previous chapters we have examined the incorporation of [^{3}H]choline into PC or [^{3}H]ethanolamine into PE and PC, and the subsequent metabolism of these labeled phospholipids. In these earlier experiments the formation of labeled-phospholipids occurred predominantly in one compartment, the endoplasmic reticulum. These experiments are uncomplicated relative to serine labeling experiments. The incorporation of [^{3}H]serine into PS, PE, and PC is much more complex in that several compartments and at least two transfer steps are involved. As mentioned in chapter I, the primary route of serine incorporation into PS is by the base exchange pathway (Hübscher, 1959). This biosynthetic step takes place in the microsomal fraction and is catalyzed by the enzyme PS synthetase (Dennis and Kennedy, 1972). For conversion to PE, the newly made PS must be transferred to the mitochondria where it can be decarboxylated by PS decarboxylase (Dennis and Kennedy, 1972). The importance of this pathway for PE synthesis in hepatocytes is unclear but it has been shown to contribute significantly in non-hepatic cell lines (Voelker, 1984, Miller and Kent, 1986). The serine-derived PE must be transferred back to the either the endoplasmic reticulum or the Golgi as the mitochondria lacks the enzyme PEMT.

Several recent studies have examined the metabolism of [^{3}H]serine in suspension cultures (Bjerve, 1985) and monolayer cultures (Vance, 1989) of rat hepatocytes, using a continuous-pulse protocol. In addition to labeling phospholipids the [^{3}H]serine-derived label may be incorporated into the one-carbon tetrahydrofolate, acetate, and methionine pools (Bremer et al., 1960). Recently, it has been demonstrated that [^{3}H]serine will be incorporated into formate, glycine and ATP, during long term incubations in the C6 glioma cell line (Xu et al., 1991). Thus, [^{3}H]serine-label will be incorporated into many catabolites during long term incubations and hence caution must be used when interpreting the results of such experiments. Because of this caveat we have employed several 'short-term' pulse-chase protocols to label cell phospholipids.

Previously, we have demonstrated that newly-made PE, derived from ethanolamine, is not significantly remodeled in the rat hepatocyte (Samborski et al., 1990; Chapter III). This finding was unexpected as it was generally accepted that hepatic PE was remodeled (Holub and Kuksis, 1978; Åkesson et al., 1976; Åkesson et al., 1970a; Åkesson et al., 1970b; Trewhella and Collins, 1973; de Tomas, and Brenner, 1970; Holub and Kuksis, 1971). Recently, Schmid et al. (1991) have demonstrated that there is substantial turnover of the fatty acyl moiety of hepatic PE. However, a major critisism of the studies of Schmid et al. (1991) is that they (a) do not distinguish between remodeling reactions and recycling of fatty acyl groups into diacylglycerides for *de novo* PE biosynthesis and (b) do not distinguish between different pools of PE in the hepatocyte. While we do not believe that the ethanolamine derived PE pool is being significantly remodeled in the hepatocyte, the serine derived PE pool may be significantly remodeled. Thus it is the purpose of this study to examine the metabolism of newly-made PE, derived from [^{3}H]serine

MATERIALS AND METHODS

Materials

The same materials were used in the studies shown in chapter II were used for these studies with the following additions: Phospholipase C (*Clostridium welchii* and *Bacillus cerus*), O-phosphoserine, L-α-glycerolphosphorylserine, serine, and diolein were from Sigma Chemical Co., St. Louis, MO, [3-^{3}H]serine (specific radioactivity of 23 Ci/mmol) was from Amersham Corp., U.K.

Animals and Hepatocytes

Same as described in chapter II with the following exception: hepatocytes were isolated and cultured in serine-free Dulbecco's MEM containing 200 µM methionine.

Pulse chase experiments

Just prior to the start of the experiment the medium was removed and replaced with serum-, serine-free MEM and the cells were equilibriated for 60 min. In the initial experiments twenty-five µCi of [3-^{3}H]serine was added to each dish in 2 ml serum-, serine-deficient MEM and the cells were incubated for 1 h. The medium was replaced with 2 ml of serum-deficient MEM containing 100 µM serine and the cells were subsequently incubated for the indicated times. In all other experiments seventy-five µCi of [3-^{3}H]serine was added to each dish in 2 ml serum-, serine-deficient MEM and the cells were incubated for 10 min. The medium was replaced with 2 ml of serum-deficient MEM containing 300 µM serine and 100 µM ethanolamine and the cells were subsequently incubated for the indicated times.

Experimental analysis

Same as described in chapter II and III with the following additions.

Head Group Analysis

Determination of distribution of radioactivity in phospholipids: [3-^{3}H]serine was incorporated into the fatty acid, glycerol, and head group moiety of the phospholipid molecules. To follow molecular species remodeling it was necessary to determine the amount of label associated with the head group of the PC, PE and PS molecules. This was done according to the phospholipase C (PLC) method of Kuksis et al. (Kuksis et al., 1981) with some modifications (Vance and Vance,1986a). Briefly, PC, PE and PS were isolated from six dishes of cells and resuspended in 1.0 ml of chloroform/methanol (2:1, v/v). An aliquot of this suspension, corresponding to approximately 50 nmol of phospholipid, was transferred to a 10 ml screw-top tube and the sample was dried under nitrogen. Stock solutions of PLC were prepared from lyophilized enzyme preparations. Typically, 125 units of PLC from *C. welchii* or 100 units of PLC from *B. cerus* were dissolved in 200 µl of Tris buffer (17.5 mM, pH 7.3). For the analysis of PC samples, 1 unit of PLC from *C. welchii* was added to 5.5 Tris buffer (13.3 mM, pH 7.3) containing 0.24% $CaCl_2$. For the analysis of PE or PS samples, 1 unit of PLC from *B. cerus* was added to 5.5 ml Tris buffer (13.3 mM, pH 7.3) containing 0.24% $CaCl_2$. These enzyme cocktails were stored on ice until needed. 1 ml of diethylether was added to the phospholipid sample followed by the addition of 5.3 ml of the appropriate enzyme cocktail. The tubes were tightly capped, placed in a shaking water bath (300-320 rpms), and incubated at 30°C for 3 h. The digestion was stopped by the addition of 0.2 ml of 0.1 N HCl and the mixture was transferred to a 50 ml conical tube. The 10 ml tubes were rinsed with 3 x 5 ml of chloroform/methanol (2:1, v/v) and the wash was transferred to the 50 ml conical tubes. The samples were vortexed, centrifuged and an aliquot of the upper aqueous phase (containing the head group) was removed and transferred to glass tubes. The aqueous fraction was dried in a Vacu-oven overnight, resuspended in 500 µl of distilled water and the radioactivity was determined. 250 µg of diolein was added to the lower phase (containing DG and unreacted phospholipid), the sample was dried and dissolved in 1.0 ml of chloroform/methanol (2:1, v/v). The radioactivity associated with 100 µl was determined and the remaining sample was dried and applied to a TLC plate. DG and phospholipid were separated by developing the TLC plates in benzene/acetic acid/ethyl acetate/diethylether (8:0.02:1:1, v/v/v/v). In this solvent system, phospholipids remained at the origin while DG migrated to the middle of the plate. The bands corresponding to phospholipid and DG were visualized with iodine vapors, scraped into scintillation vials and counted. Typically, the head group accounted for 91%, 49%, and 15% of the total phospholipid radioactivity in PS, PE, and PC, respectively. The total specific activity of PE and PC molecular species were determined as described in chapter II. These values were then corrected for % label in the head group and presented as head group specific activity.

Aqueous [^{3}H]Serine Catabolites

For analysis of aqueous serine catabolites the following was routinely done. To an aliquot of the upper phase (usually 100-250 μl) 0.4 μmole ethanolamine (E) and serine (S) and 1 μmole each of ethanolamine-phosphate (E-P), serine-phosphate (S-P), glycerolphosphoethanolamine (GPE) and glycerolphosphoserine (GPS) were added as carriers and the sample was dried under a stream of air. The sample was dissolved in 100 μl of methanol/H_2O (1:1, v/v) and 50 μl was analyzed by TLC on silica gel 60. The plate was developed in methanol/0.6% NaCl/isopropylether (6:4:1, v/v/v) and the bands corresponding to E + E-P, S-P, S, GPE, and GPS were visualized with ninhydrin (2.5% in acetone), scraped into scintillation vials and counted. The data was corrected to reflect recoveries which ranged from 75-90%. In this solvent system E and E-P co-chromatographed but together they never contained greater than 3% of total aqueous radioactivity therefore further separation was deemed unnecessary. Typically E + E-P, S-P, S, GPE, and GPS had R_f values of 0.35, 0.45, 0.60, 0.68 and 0.73, respectively. In this same system glycine acetate and methionine had R_f values of 0.56, 0.85, and 0.70, respectively.

RESULTS

Metabolism of [^{3}H]Serine in Hepatocytes

In preliminary studies, cultured rat hepatocytes were incubated for 1 h with 25 μCi of [3-^{3}H]serine and chased for up to 24 h with 100 μM serine. At the end of the pulse approximately 1% of the [3-^{3}H]serine radiolabel had been incorporated into phospholipid. Similar [^{3}H]serine incorporation has previously been described in suspension cultures of rat hepatocytes (Bjerve, 1985). Of the total radiolabel associated with phospholipids at the end of the pulse, 62%, 13%, and 25% was associated with PS, PE, and PC, respectively. It was calculated that during the initial 2 h of the chase period only 30% of the label lost from the PS head group appeared in PE (28.6%) and PC (1.4%). Surprisingly, 70% of label lost from PS during this period reflected degradation reactions and not further metabolism. This finding suggests that only a portion of the newly-made PS is transferred to the mitochondria for further processing and if not transferred is rapidly catabolized.

From measurements of the masses of the phospholipids, we were able to calculate the specific radioactivity of [3-^{3}H]serine-labeled PS, PE, and PC (Fig. VI.1.). During the chase period the specific activity of PS decreased in an exponential manner while the specific activity of PE and PC increased. The specific activity of [3-^{3}H]serine-labeled PE continued to increase during the 24 h chase period. This finding was contrasted with the rapid turnover of [^{3}H]ethanolamine-labeled PE described in chapter II and complicated our attempts to study the potential remodeling of PE molecular species. Interestingly, [3-^{3}H]serine-labeled PS, similar to [^{3}H]ethanolamine-labeled PE, was rapidly catabolized. As both of these phospholipids are synthesized on the endoplasmic reticulum, the phospholipase A, responsible for the degradation of [^{3}H]ethanolamine-labeled PE (Samborski et al., Chapter III.), may be implicated in the degradation of newly-synthesized PS. In contrast [3-^{3}H]serine-labeled PE, synthesized in the mitochondria and therefore not immediately accessible to the endoplasmic reticulum PLA, has a slow turnover. Alternatively, PS may be degraded by other routes (ie PLC, PLD, or by reversal of PS synthetase). A final observation from these studies was that, unlike [^{3}H]ethanolamine-labeled PE, [3-^{3}H]serine-labeled PE was not a very good substrate for PEMT. This observation is in agreement with our earlier finding that newly-made PE, via the CDP-ethanolamine pathway, was a preferred substrate for the PEMT relative to bulk PE (Samborski et al., 1990).

Using the 1 h pulse protocol, in these preliminary studies, we were unable to chase label out of total PE or individual PE molecular species (results not shown). As with earlier choline experiments, this result probably reflected the labeling of a large precursor pool. Due to this problem we could not determine if remodeling of PS-derived PE molecular species was occurring. Thus, we decided to change our protocol such that the cells were pulsed very quickly with a large amount of [3-^{3}H]serine and then chased for a shorter time period.

In the subsequent experiments cultured rat hepatocytes were incubated for 10 min with 75 µCi of [3-^{3}H]serine and chased for up to 4 h with 300 µM serine and 100 µM ethanolamine. During the chase period the amount of radioactivity present in the aqueous and organic fractions of cells and medium was determined (Fig. VI.2.). As expected, the [3-^{3}H]serine was poorly incorporated into cell aqueous and organic phases at the end of a 10 min pulse period. We calculated that of the radiolabel in the pulse medium, 7.4 ± 1.4% was taken up by the cell. The majority of the radiolabel was in the aqueous phase and lipid associated label accounted for less than 0.1% of the 75 µCi pulse. During the chase period the amount of label in the organic phase remained relatively constant. In contrast, during the first 15 min of the chase 71% of cellular aqueous-associated label was released into the medium. During the 15 to 240 min chase period only a small amount of radiolabel was further lost from the cell.

To determine the nature of the aqueous serine catabolites we analyzed the organic and aqueous components from both the cells and the medium of the experiments depicted in Fig. VI.2. (Table VI.1). At the end of the pulse nearly 80% of cell radiolabel was with serine, while surprisingly 11% was associated with the GPE fraction. After 15 min of chase the serine and the GPE fraction accounted for 41% and 40% of total cell label, respectively. The GPE fraction was subjected to acid hydrolysis and the amount of radiolabel associated with the ethanolamine group was determined after separation by TLC. This analysis demonstrated that GPE accounted for less than 5% of the label in this fraction. In an attempt to identify the source of this radiolabel, methionine, glycine and acetate (known catabolites of serine) were chromatographed with serine, GPS, and GPE, in the solvent system methanol/0.6% NaCl/isopropylether (6:4:1, v/v/v). In this system serine, glycine, GPE, methionine, GPS, and acetate had R_f values of 0.60, 0.60, 0.68, 0.70, 0.73, and 0.85, respectively. Thus it appears that the label in the GPE fraction was probably labeled methionine. Methionine may have also been a contaminant of the GPS fraction. As the hepatocytes were cultured in medium containing 200 µM of methionine, minimal radiolabeled methionine would be incorporated into PC via PE methylation.

While the analysis of ethanolamine catabolites revealed that PE was mainly catabolized by a PLA activity (Samborski et al., 1990), the analysis of serine catabolites does not indicate any specific route of PS degradation. Any specific route of catabolism is obscured since the radiolabel lost from the PS pool is insignificant in comparison to the label in the aqueous pool. Furthermore, our analysis was also complicated by the inability to separate completely methionine from GPE and GPS. Thus, we are unable to comment on the route of PS degradation in these experiments.

The specific radioactivity of [3-^{3}H]serine-labeled PS, PE, and PC, isolated from the experiments depicted in Fig. VI. 2., was determined (Fig. VI.3.). During the chase period the specific activity of PS decreased in an exponential manner. The specific activity of PE increased during the first 30 min of the chase and then remained relatively constant for the remaining 210 min of the chase ($0.1<p<0.375$). Thus, during the 30 to 240 min period we could examine the metabolism of PE molecular species. The specific radioactivity of PC also increased during the first 30 min and then remained constant.

In addition to the head group, [^{3}H]serine will also be incorporated into the glycerol and fatty acid moieties of the phospholipid. The distribution of radiolabel in the head group of PS, PE, and PC was determined after PLC digestion as described in the Materials and Methods (Table VI.2.). After a 10 min pulse the head groups of PS, PE, and PC contained 91%, 49%, and 15% of the total phospholipid label, respectively. During the following 4 h of chase these values did not change significantly. A comparison of these results to those recently reported (Vance, 1989; Bjerve, 1985), indicate that these values are remarkably similar for PE and PS. There appears to be some variation in the values reported for PC. These differences probably reflect differences in culture conditions.

The specific radioactivity of [^{3}H]serine-labeled phospholipids in Fig. VI.3., was corrected for the percent label in the head group moiety, using the data in Table VI.2., and is shown in Fig. VI.4. This analysis indicates that only a small portion of the PS head group is incorporated into PE, and that even less of the label is incorporated into the PC head group. Thus, apparently very little serine-labeled PE was methylated. This supports our previous finding that for the most part only newly-synthesized PE is used by the PE methyltransferase (Samborski et al., 1990). These results suggest that PS decarboxylation followed by PE methylation is not a major pathway of PC biosynthesis in the rat liver. It might be argued that the presence of 100 μM ethanolamine in the chase medium would reduce the flux of serine-label into PC. However, Tijburg et al. (1989) have recently demonstrated, in similar experiments, that the inclusion of 100 μM ethanolamine in the chase medium does not affect the incorporation of [^{3}H]serine label into the PE head group. These researchers neglected to examine PC in their studies.

Metabolism of [^{3}H]Serine-Labeled PE Molecular Species

The [^{3}H]serine-labeled PE, isolated from the experiments depicted in Fig VI.2., was fractionated into molecular species by HPLC. The percent distribution of label in PE molecular species is shown in Fig. VI.5. At the end of the pulse docosahexaenoate and arachidonate-containing species accounted for 32% and 29% of the incorporation of [^{3}H]serine into PE. This finding is in agreement with an earlier study (Bjerve, 1985) which demonstrated that the docosahexaenoate- and arachidonate-containing species accounted for 48% and 33% of the incorporation of [^{3}H]serine into PE. The major species of PE labeled at the end of the pulse was 16:0-22:6-PE and 18:0-20:4-PE, which accounted for 23% and 22% of the incorporation of [^{3}H]serine into PE. During the 4 h chase period, the percent distribution in 16:0-22:6-PE decreased to 15%, closely approaching the mole % of this species in cellular PE (Samborski et al., 1990). Concomitantly, the percent incorporation of label into 18:0-20:4-PE increased over the 4 h chase period from 22 % to 32 %. Three other molecular species were very active during the chase period. The percent label in 16:0-20:4-PE and 16:0-18:2-PE both increased approximately 2-fold. Interestingly, 16:0-18:1-PE increased from 5.3% to 18.1% in the first 30 min of the chase, and then in the subsequent 30 to 240 min period decreased to 8%. A recent study has examined the % distribution of [^{3}H]serine into PE, after a 30 min labeling period, and is in complete agreement with the data presented here (Vance, 1989). Thus, in contrast to the metabolism of [^{3}H]ethanolamine-labeled PE, [^{3}H]serine-labeled PE appears to undergo a series of deacylation-reacylation reactions that resulted in the conversion of certain PE (peaks 7 and 12) species into others (peaks 8,9 and 14).

Previously, we demonstrated that the examination of % distribution data alone could lead to erroneous conclusions (Samborski et al., 1990). Thus, the head group specific radioactivities of the major molecular species of PE were determined as described in the Materials and Methods (Fig VI.6.). Initially, the highest specific activity was observed in peak 16:0-22:6-PE (18:0-22:5$_{(n-3)}$-PE also had a high specific activity but, because it accounts for only 3% of the mass of PE, it is probably not significant). During the first 15 min of the chase the specific activity in all PE species increased. These increases were most dramatic for 16:0-22:6-PE and 16:0-18:1-PE, which showed a 3.2-fold and 7.1-fold increase, respectively. The specific activity of 16:0-22:6-PE did not increase after 15 min of chase. The specific activity of 16:0-18:1-PE reached a maximum at 30 min and then decreased by 50% during the 30 to 240 min chase period. The specific activity of some other molecular species (peaks 10, 11 13, and 17) reached a plateau by 15 min of the chase. Interestingly, the specific activity of 16:0-20:4-PE, 16:0-18:2-PE and 18:0-18:2-PE reached a plateau at 90 min of the chase while 18:0-20:4-PE continued to increase through out the chase.

Taken together, the data in Fig. VI.5 and Fig. VI.6. suggest that, in contrast to the metabolism of [^{3}H]ethanolamine-labeled PE, [^{3}H]serine-labeled PE undergos a series of deacylation-reacylation reactions that resulted in the conversion of certain PE (peaks 7 and 12) species into others (peaks 8,9 and 14). Previously, we have demonstrated that 16:0-18:1-PC and 16:0-18:2-PC were extensively converted to 18:0-20:4-PC and 18:0-18:2-PC species (Tijburg et al., 1991). Thus, in terms of remodeling reactions, the metabolism of [^{3}H]serine-labeled PE molecular species resembles that of [^{3}H]choline-labled PC species.

Metabolism of [^{3}H]Serine-Labeled PC Molecular Species

The [^{3}H]serine-labeled PC, isolated from the experiments depicted in Fig VI.2., was fractionated into molecular species by HPLC. The percent distribution of label in PC molecular species is shown in Fig. VI.7. A comparison of the data in Fig. VI.5. and Fig.VI.7. indicate that, unlike [^{3}H]ethanolamine-labeled PE and PC, the % distribution of [^{3}H]serine-labeled PC differed significantly from the corresponding data for PE at the end of the chase. The [^{3}H]serine-labeled PC resembled more closely the mol% distribution of cell PC (Table III.3.) with the exception of 16:0-18:1-PC. Overall, these result are in close agreement with an earlier study (Vance, 1989) The major species of PE labeled at the end of the pulse was 16:0-18:1-PC and 18:0-20:4-PC, which accounted for 14% and 19% of the incorporation of [^{3}H]serine into PC. During the 4 h chase period, the percent distribution of label in all of the species remained constant. Examination of this data did not indicate the occurrence of remodeling reactions.

The head group specific radioactivities of the major molecular species of PC were determined as described in the Materials and Methods (Fig VI.8.). Initially, no species was predominant but as the chase period progressed the specific activity in the palmitate-containing species increased at a greater rate than the corresponding stearate-containing species. As the specific activities of all the PC species increased it is concluded that synthesis and not remodeling accounted for the increase in specific activities. Thus, unlike PC derived from [^{3}H]choline or [^{3}H]ethanolamine, the PC derived from [^{3}H]serine undergoes very little observable remodeling.

DISCUSSION

Lack of Remodeling of the Molecular Species of [^{3}H]Serine-Labeled PC

It has become well recognized that cellular phospholipids may be remodeled by deacylation and reacylation events, a phenomenon described by Lands nearly 25 years ago (Lands, 1965). We demonstrated in chapter III that PC, derived from [^{3}H]ethanolamine, was rapidly remodeled in normal hepatocytes. Interestingly, the present experiments failed to show that remodeling of [^{3}H]serine-derived PC occurs in cultured hepatocytes. We have previously demonstrated that the remodeling of [^{3}H]choline-derived PC is somewhat obscured in normal rat hepatocytes (Samborski et al., 1990) and only becomes evident when choline-deficient hepatocytes were used (Tijburg et al., 1991). Thus it is possible that the remodeling of PC, derived from serine, is simply obscured by continuous synthesis. To test this hypothesis we could inhibit the enzyme PEMT with the drug 3-deazaadenosine (DZA), and examine the metabolism of [^{3}H]serine-labeled PC molecular species in the absence of continued synthesis. DZA is converted to 3-deaza-adenosylhomocysteine, a strong inhibitor of *S*-adenosylhomocysteine hydrolase, which causes a rise in the cellular levels of *S*-adenosylhomocysteine and decreases the levels of *S*-adenosylmethionine in rat hepatocytes (Chiang and Cantoni, 1979). PEMT is especially sensitive to the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine (Hoffman et al., 1980) and is therefore rapidly inhibited by DZA-treatment (Pritchard et al., 1982). Unfortunately these studies were not done.

[^{3}H]serine-derived label may be incorporated into the one-carbon tetrahydrofolate, acetate, and methionine pools (Bremer et al., 1960). However, Bjerve (1985) has demonstrated that the *S*-adenosylmethionine pool is not a significant route for the incorporation of labeled serine into the PC head group, and that the major route for the incorporation of [^{3}H]serine label into PC is by the methylation of [^{3}H]serine labeled PE. While the methylation of serine- and ethanolamine-derived PE is generally thought to ocurr by the same mechanism there is some evidence which suggests otherwise. Vance and Vance (1986b) have demonstrated that the methylation of serine-derived PE was partially insensitive to DZA. While DZA inhibited the incorporation of [^{3}H]serine and [^{3}H]ethanolamine into cellular PC, DZA had no effect on the incorporation of [^{3}H]serine into PC which was being secreted (Vance and Vance, 1986b). Furthermore, it has been demonstrated that serine-derived PC is preferentially secreted by hepatocytes (Vance and Vance, 1986a). Thus, it is possible that the [^{3}H]serine-labeled PC is synthesized at a separate location from [^{3}H]ethanolamine-labeled PC and is not accessible to the remodeling enzymes.

Remodeling of the Molecular Species of [^{3}H]Serine-Labeled PE

The remodeling of PE was somewhat surprising, particularly since we had found little evidence for it occurring in [^{3}H]ethanolamine-labeled PE (Samborski et al., 1990; Chapter III.). However, previously we had speculated that PE, made by decarboxylation of phosphatidylserine, could be modified significantly by remodeling reactions even though ethanolamine-derived PE is not remodeled (Samborski et al., 1990). From Fig. VI.6, it appears that 16:0-18:1-PE is being converted to other molecular species, in particular 18:0-20:4-PE. In such transformations the newly-formed 16:0-18:1-PE would have both its *sn*-1 and *sn*-2 fatty acids replaced by the classical deacylation-reacylation pathway (Lands, 1965). However, while it is generally accepted that remodeling of phospholipids can occur at both the *sn*-1 and *sn*-2 position (Holub, 1980; Thompson and Belina, 1986) it is assumed that remodeling of PC mainly involves *sn*-2 remodeling (MacDonald and Sprecher, 1991).

This conversion may begin with deacylation of palmitate-containing species by phospholipase A_2, followed by reacylation of the 1-acyl-glycerophosphocholine. The acylCoA: lysophospholipid acyltransferases, in general, are usually localized in the microsomal fraction of most tissues (Severson and Fletcher, 1985) but have been demonstrated in the mitochondrial fraction as well (Arthur and Choy, 1986). Thus the lysoPE could be reacylated by this enzyme either in the mitochondria or in the endoplasmic reticulum. Several studies have indicated that arachidonic acid enters rat liver PC mainly by acylation of 1-acyl-sn-glycerophosphocholine (Kanoh, 1969; Holub and Kuksis, 1971; Kanoh and Ohno, 1975). It is not known if this is true for PE.

Remodeling of the *sn*-1 position initially requires the action of a phospholipase A_1. Since phospholipase A_1 is predominantly located in the microsomes, lysosomes and cytosolic fraction but not the mitochondria (Waite, 1987), the *sn*-1 remodeling of newly-made PE would require transport from the mitochondria to an organelle containing PLA_1. Alternatively, the serine-derived PE could be on the outer membrane of the mitochondria where it would be accessible to a cytosolic phospholipase A_1. The presence of a PLA_1 in the cytosol of rat liver has been known for at least 25 years (Waite and van Deenen, 1967) and has been recently described in rat brain (Doherty and Rowe, 1980), rat liver (Dawson et al. 1983), and rat heart (Nalbone and Hostetler 1985). The reacylation of the 2-arachidonyl-glycerophosphoethanolamine may occur by a CoA-dependent pathway. Åkesson et al. (1970) provided *in vivo* evidence to support the preferential reacylation of 2-linoleoyl-sn-glycerophosphocholine with stearic acid. Thus, stearate may be incorporated into the lysoPE molecule via an acyl-CoA:2-acyl-glycerophosphoethanolamine acyltransferase. Alternatively, stearate may be incorporated into the *sn*-1 position via the action of CoA-dependent transacylase system present in liver microsomes (Sugiure et al., 1988). The exact contribution that these two pathways make towards stearate incorporation is unknown.

The CoA-independent transacylation pathway has been described in many tissues (reviewed in MacDonald and Sprecher, 1989), but typically this pathway involves the transfer of an acyl group from a donor phospholipid, usually a diacyl-glycerophosphocholine, to an acceptor lysophospholipid, usually either a 1-alkyl- or 1-alkenyl-phospholipid. Consequently, this pathway is probably not involved in the remodeling of hepatic PE. Thus, the conversion of 16:0-18:1-PE to 18:0-20:4-PE appears to involve a multi-step remodeling reaction which may take place in both the endoplasmic reticulum (ER) and the mitochondria. While it has recently been suggested that the majority of rat liver mitochondria PLA_2 may be derived from Kuppfer cells and not hepatocytes (Wilton et al., 1991) the absence of a PLA_2 in rat liver mitochondria remains to be demonstrated. Until this is demonstrated we can not exclude involvement of the mitochondria in remodeling reactions.

Why is [^{3}H]serine-labeled PE but not [^{3}H]ethanolamine-labeled PE remodeled? An obvious answer is that the site of synthesis is probably responsible for these different metabolic fates. The [^{3}H]serine labeled PE is formed in the mitochondria and may be protected from PLA degradation which accounts for the high turnover of [^{3}H]ethanolamine-labeled PE (Samborski et al., 1990). It is possible that both [^{3}H]serine labeled PE and [^{3}H]ethanolamine-labeled PE are deacylated in vivo but that the lysoPE intermediate, generated during the deacylation steps, may be more stable in the mitochondria than in the microsomes and therefore more readily reacylated. In support of this is the observation that the rat liver mitochondrial fraction has little lysophospholipase activity towards lysoPE (de Winter et al., 1982) while the microsomal fraction actively hydrolyzes lysoPE (Holub, 1982; Pind, 1985). In addition, during the reacylation step the microsomal lysoPE will have to compete for acyl-CoA's with microsomal lysoPC. It has been shown that 1-acyl-lysoPC is reacylated at a much higher rate than 1-acyl-lysoPE (Funkhouser et al, 1981). Thus the lysoPE may not be rapidly reacylated but rather left exposed to the microsomal lysophospholipase. Such competition apparently does not exit in the mitochondria due to the low lysophospholipase activity present in the mitochondria (de Winter et al., 1982; Fig. VI.8.).

Another very important observation is that newly-made 16:0-22:6-PE (Fig. VI.6.), derived from serine, in contrast to newly-made16:0-22:6-PE (Samborski et al., 1990) derived from ethanolamine, does not turnover very rapidly. This indicates that the enzyme(s) responsible for the catabolism of ethanolamine-derived PE species are not located throughout the cell but are found in close association with the enzymes of the CDP-ethanolamine pathway.

The lack of remodeling of ethanolamine-derived PE in hepatocytes is contrary to the view that has prevailed over the past two decades (Holub and Kuksis, 1978; Åkesson et al., 1976; Åkesson et al., 1970; Åkesson, 1970; Trewhella and Collins, 1973; de Tomas, and Brenner, 1970; Holub and Kuksis, 1971). Previously, we reviewed this earlier literature and showed that the evidence for remodeling of PE molecular species was not conclusive and that the experimental approaches used were archaic and flawed (Samborski et al., 1990). Using cultured hepatocytes, short-term pulse-chase experiments, and HPLC analysis of phospholipid molecular species we have been able to demonstrate that while ethanolamine-derived PE is not significantly remodeled (Samborski et al., 1990), serine-derived PE appears to be somewhat remodeled. This observation clearly indicates that, with respect to PE metabolism, there are at least two separate pools of newly-made PE in the hepatocyte. This finding is supportive of the previous finding that there are multiple pools of phospholipids within the hepatocytes (Vance and Vance, 1986a).

FIGURE VI.1.

SPECIFIC RADIOACTIVITY OF CELLULAR PS, PE, AND PC DERIVED FROM [^{3}H]SERINE

Hepatocytes were prepared as described in the Materials and Methods. At the start of the experiment medium was replaced with fresh MEM (serine-deficient) and incubated for 60 min. The medium was then replaced with 2 ml serine-deficient MEM containing 12.5 µCi/ml [3-^{3}H]serine and incubated for 1 h. At the end of the pulse the medium was replaced with MEM containing 100 µM serine. At the indicated times PS. PE, and PC was isolated from a dish of cells and analyzed for radioactivity and phosphorus content as described under Materials and Methods. Values are the mean of four separate experiments ± S.D.

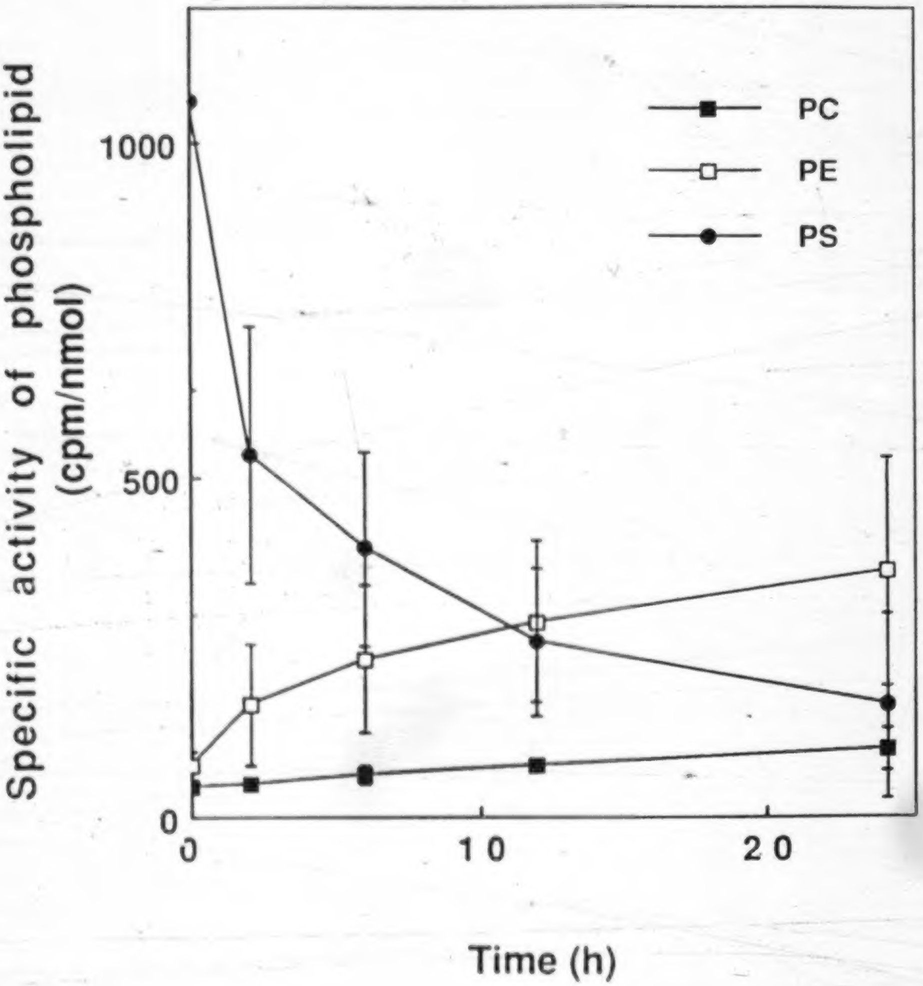
Specific activity of phospholipid (cpm/nmol)
1000
500
0
0
10
20
Time (h)
PC
PE
PS

FIGURE VI.2.

DISTRIBUTION OF [^{3}H]SERINE IN CELL AND MEDIUM OF HEPATOCYTES

Hepatocytes were prepared as described in the Materials and Methods. At the start of the experiment medium was replaced with fresh MEM (serine-deficient) and incubated for 60 min. The medium was then replaced with 2 ml serine-deficient MEM containing 37.5 µCi/ml [3-^{3}H]serine and incubated for 10 min. At the end of the pulse the medium was replaced with MEM containing 300 µM serine.and 100 µM ethanolamine. At the indicated times total radioactivity associted with cell aqueous, cell organic, and medium aqueous phases was determined as described under Materials and Methods. Values are the mean of four separate experiments ± S.D.

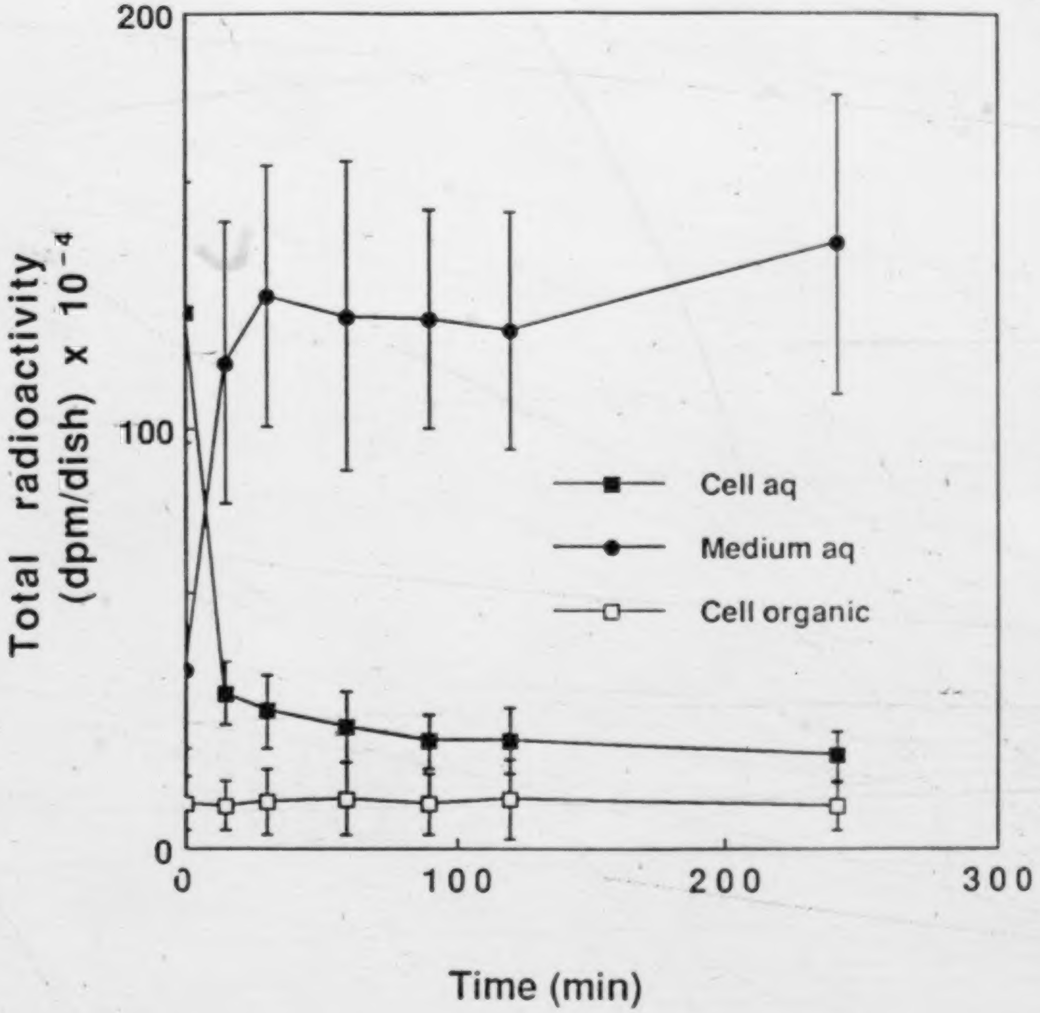
200
100
0
0
100
200
300
Total radioactivity (dpm/dish) x 10⁻⁴
Time (min)
Cell aq
Medium aq
Cell organic

FIGURE VI.3.

SPECIFIC RADIOACTIVITY OF CELLULAR PS, PE, AND PC DERIVED FROM [^{3}H]SERINE

Hepatocytes were prepared as described in the Materials and Methods. At the start of the experiment medium was replaced with fresh MEM (serine-deficient) and incubated for 60 min. The medium was then replaced with 2 ml serine-deficient MEM containing 37.5 µCi/ml [3-^{3}H]serine and incubated for 10 min. At the end of the pulse the medium was replaced with MEM containing 300 µM serine and 100 µM ethanolamine. At the indicated times PS, PE, and PC were isolated from 6 dishes of cells and analyzed for radioactivity and phosphorus content as described under Materials and Methods. Values are the mean of three separate experiments ± S.E.M.

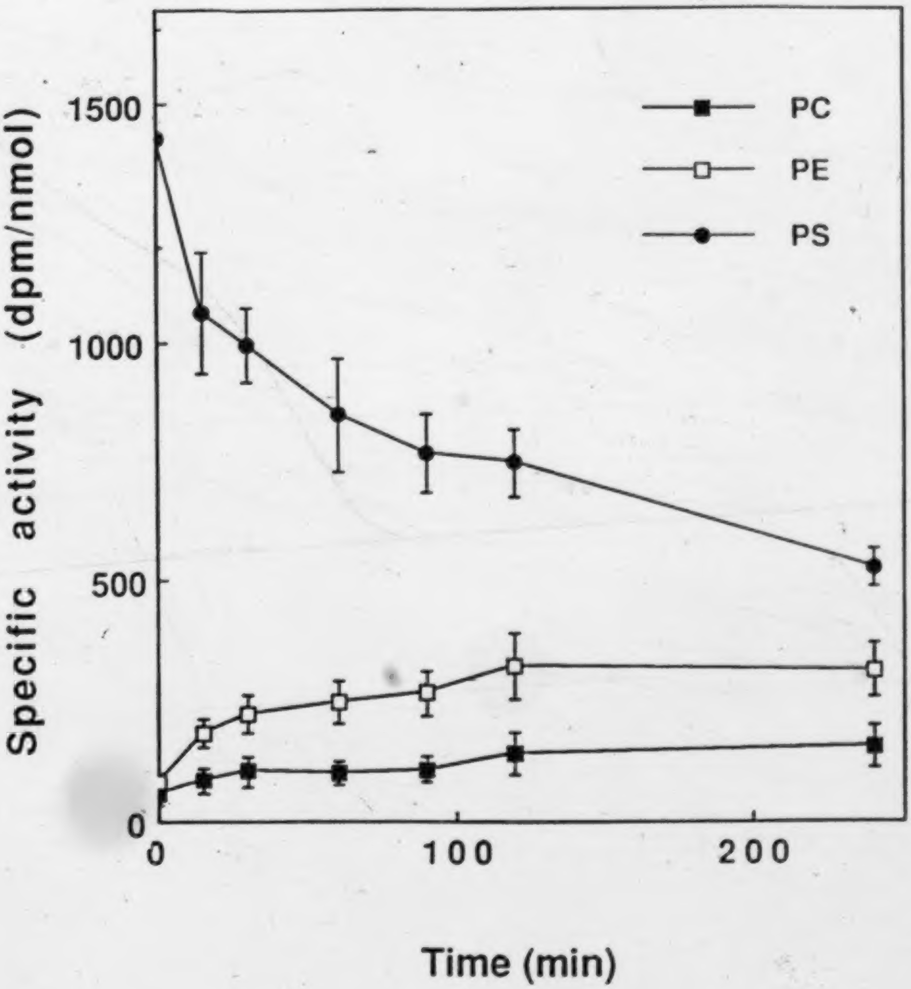
Specific activity (dpm/nmol)
1500
1000
500
0
0
100
200
Time (min)
PC
PE
PS

FIGURE VI.4.

SPECIFIC RADIOACTIVITY OF HEAD GROUP OF CELLULAR PS, PE, AND PC DERIVED FROM [^{3}H]SERINE

Hepatocytes were prepared as described in the Materials and Methods. At the start of the experiment medium was replaced with fresh MEM (serine-deficient) and incubated for 60 min. The medium was then replaced with 2 ml serine-deficient MEM containing 37.5 µCi/ml [3-^{3}H]serine and incubated for 10 min. At the end of the pulse the medium was replaced with MEM containing 300 µM serine and 100 µM ethanolamine. At the indicated times PS, PE, and PC were isolated from 6 dishes of cells and analyzed for head group associated radioactivity and phosphorus content as described under Materials and Methods. Values are the mean of three separate experiments ± S.E.M.

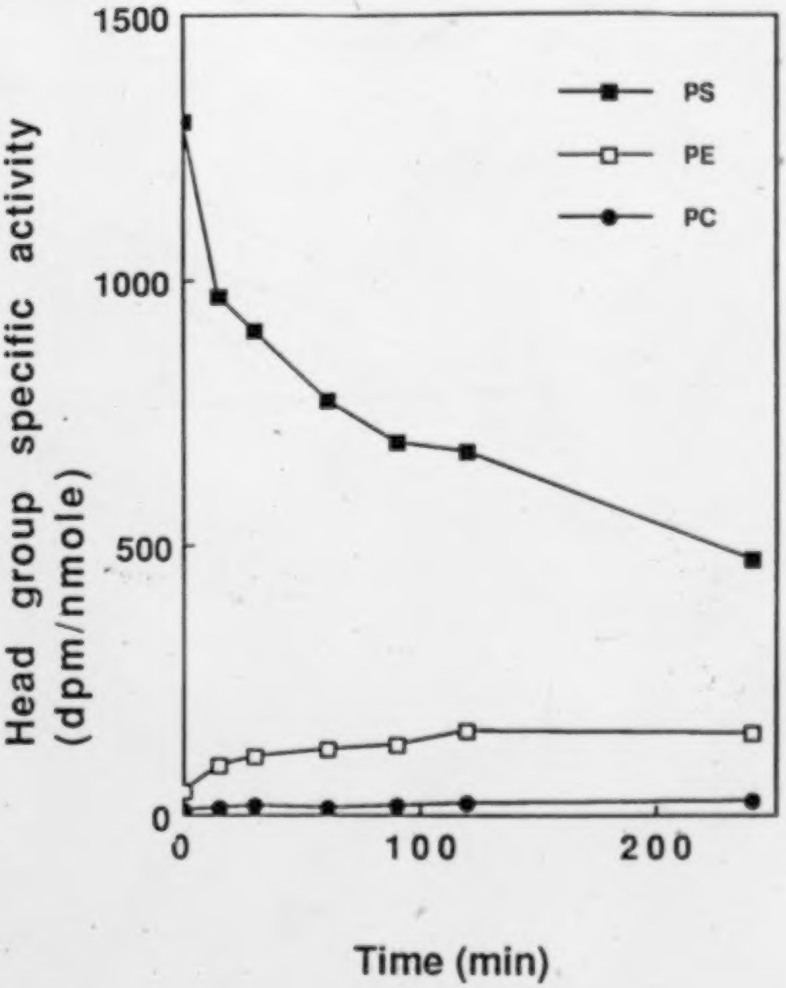
1500
1000
500
0
Head group specific activity (dpm/nmole)
0
100
200
Time (min)
PS
PE
PC

FIGURE VI.5

PERCENT DISTRIBUTION OF RADIOACTIVITY OF MOLECULAR SPECIES OF CELLULAR PE DERIVED FROM [^{3}H]SERINE

Molecular species of PE, isolated from the experiments depicted in Figure VI.2., were analyzed for radioactivity as described under Materials and Methods. Values are the mean of three spearate experiments ± S.E.M. The number on the x axis refer to the molecular species (7, 16:0-22:6; 8, 16:0-20:4; 9, 16:0-18:2; 10, 18:1-18:2; 11, 18:0-20:5 and 16:0-22:5; 12, 16:0-18:1; 13, 18:0-22:6; 14, 18:0-20:4; 15 18:0-18:2; 16, 18:0-22:5 (n-3); 17, 18:0-22:5 (n-6)).

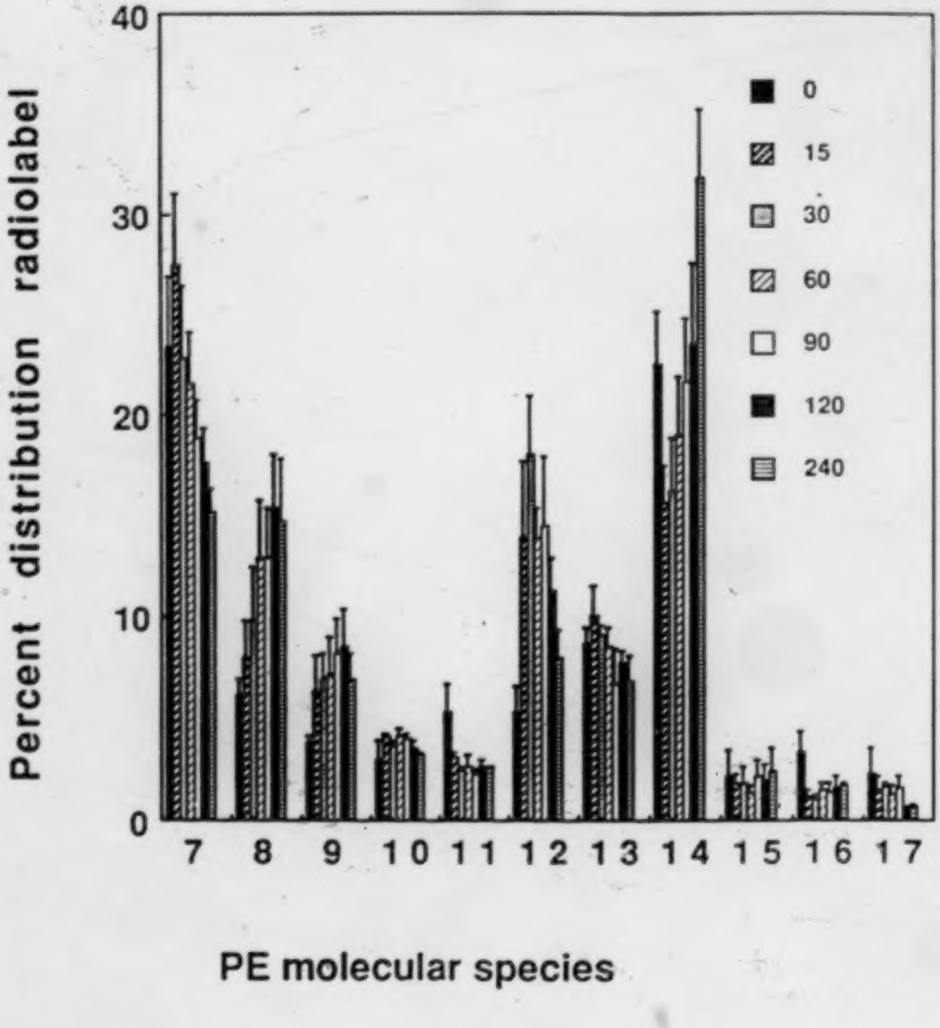
Percent distribution radiolabel
40
30
20
10
0
0
15
30
60
90
120
240
7
8
9
10
11
12
13
14
15
16
17
PE molecular species

FIGURE VI.6.

SPECIFIC RADIOACTIVITY OF HEAD GROUP OF MOLECULAR SPECIES OF CELLULAR PE DERIVED FROM [^{3}H]SERINE

Molecular species of PE, isolated from the experiments depicted in Figure VI.2., were analyzed for phosphorus and head group radioactivity as described under Materials and Methods. Values are the mean of three spearate experiments ± S.E.M. The number on the x axis refer to the molecular species (7, 16:0-22:6; 8, 16:0-20:4; 9, 16:0-18:2; 10, 18:1-18:2; 11, 18:0-20:5 and 16:0-22:5; 12, 16:0-18:1; 13, 18:0-22:6; 14, 18:0-20:4; 15 18:0-18:2; 16, 18:0-22:5 (n-3); 17, 18:0-22:5 (n-6)).

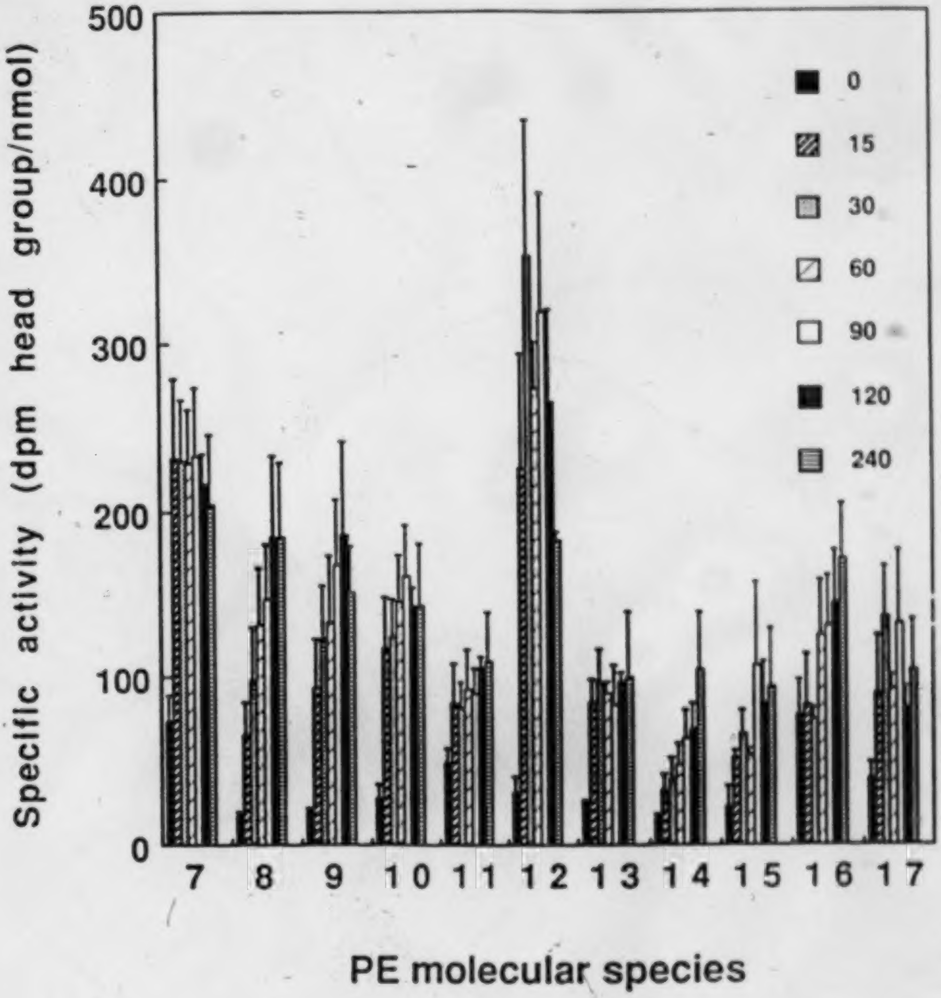
Specific activity (dpm head group/nmol)
500
400
300
200
100
0
0
15
30
60
90
120
240
7
8
9
10
11
12
13
14
15
16
17
PE molecular species

FIGURE VI.7.

PERCENT DISTRIBUTION OF RADIOACTIVITY OF MOLECULAR SPECIES OF CELLULAR PC DERIVED FROM [^{3}H]SERINE

Molecular species of PC, isolated from the experiments depicted in Figure VI.2., were analyzed for radioactivity as described under Materials and Methods. Values are the mean of three spearate experiments ± S.E.M. The number on the x axis refer to the molecular species (7, 16:0-22:6; 8, 16:0-20:4; 9, 16:0-18:2; 10, 18:1-18:2; 11, 18:0-20:5 and 16:0-22:5; 12, 16:0-18:1; 13, 18:0-22:6; 14, 18:0-20:4; 15 18:0-18:2; 16, 18:0-22:5 (n-3); 17, 18:0-22:5 (n-6)).

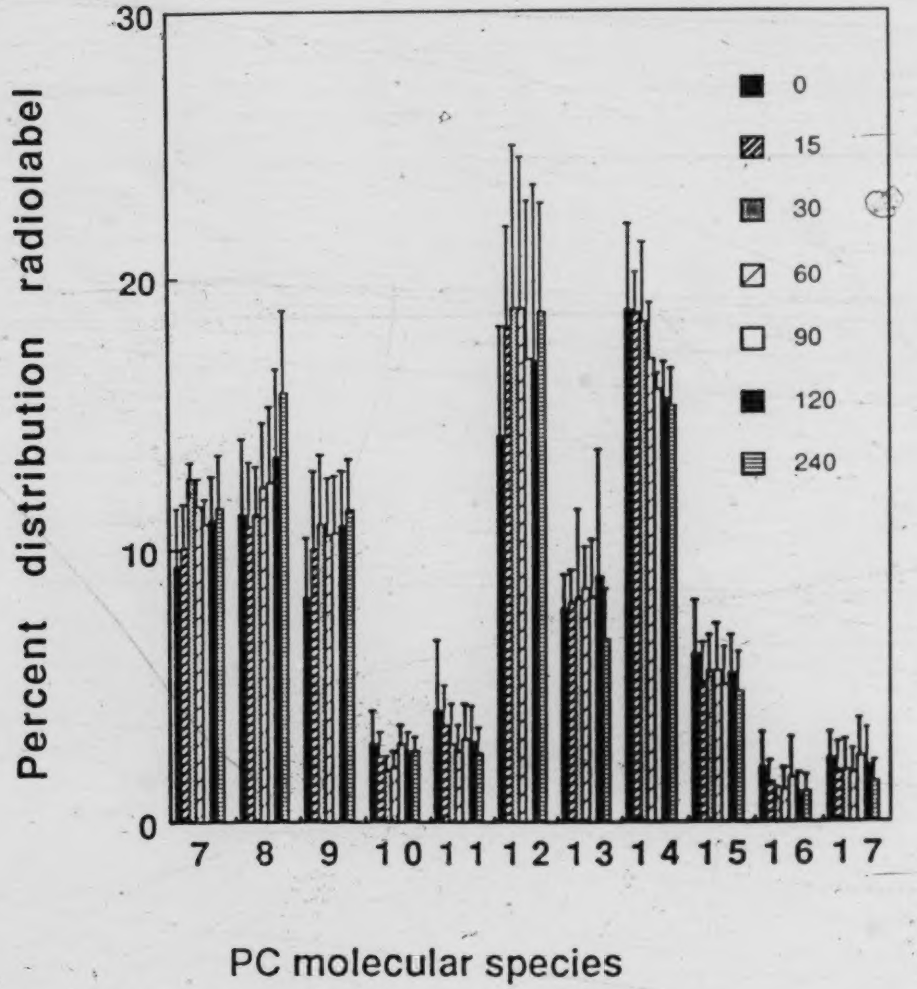
Percent distribution radiolabel
0
10
20
30
0
15
30
60
90
120
240
7
8
9
10
11
12
13
14
15
16
17
PC molecular species

FIGURE VI.8.

SPECIFIC RADIOACTIVITY OF HEAD GROUP OF MOLECULAR SPECIES OF CELLULAR PC DERIVED FROM [^{3}H]SERINE

Molecular species of PC, isolated from the experiments depicted in Figure VI.2., were analyzed for head group radioactivity and phosphorus as described under Materials and Methods. Values are the mean of three spearate experiments ± S.E.M. The number on the x axis refer to the molecular species (7, 16:0-22:6; 8, 16:0-20:4; 9, 16:0-18:2; 10, 18:1-18:2; 11, 18:0-20:5 and 16:0-22:5; 12, 16:0-18:1; 13, 18:0-22:6; 14, 18:0-20:4; 15 18:0-18:2; 16, 18:0-22:5 (n-3); 17, 18:0-22:5 (n-6)).

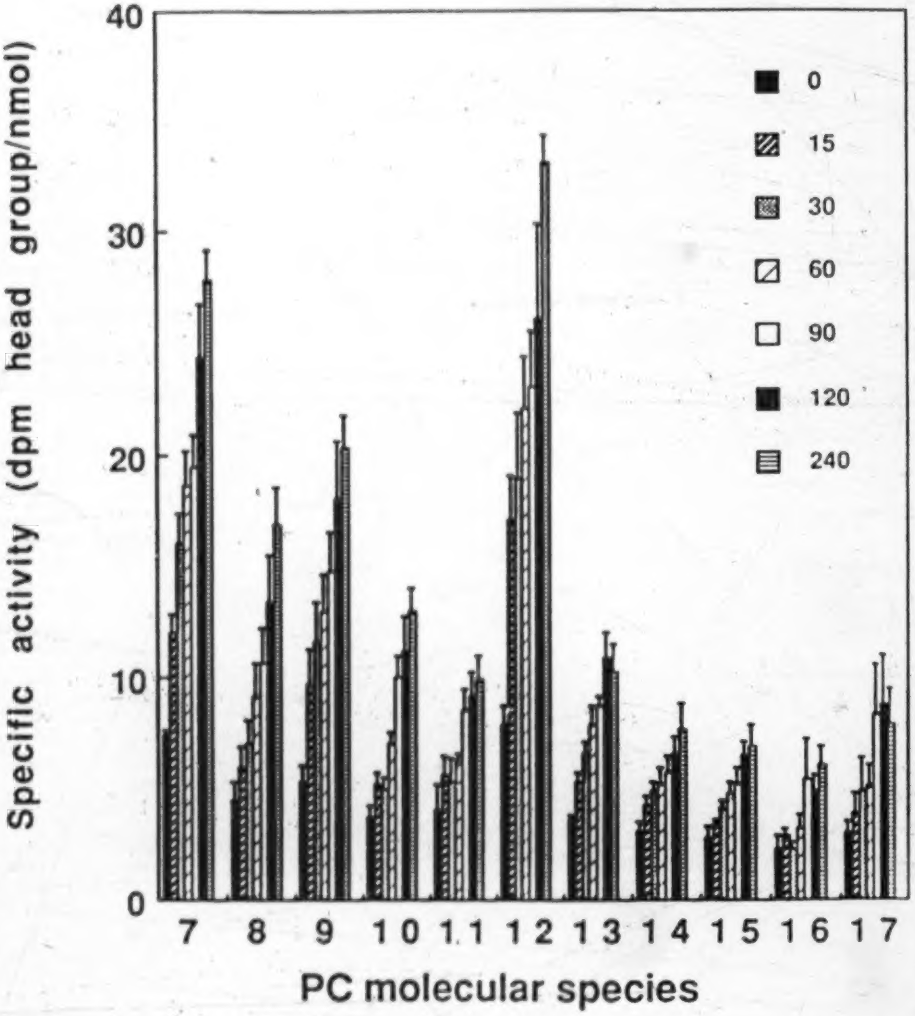
Specific activity (dpm head group/nmol)
40
30
20
10
0
0
15
30
60
90
120
240
7
8
9
10
11
12
13
14
15
16
17
PC molecular species

TABLE VI.1.

CATABOLISM OF [^{3}H]SERINE DURING A 4-H CHASE

Hepatocytes were prepared as described in the Materials and Methods. At the start of the experiment medium was replaced with fresh MEM (serine-deficient) and incubated for 60 min. The medium was then replaced with 2 ml serine-deficient MEM containing 37.5 µCi/ml [3-^{3}H]serine and incubated for 10 min. At the end of the pulse the medium was replaced with MEM containing 300 µM serine,.and 100 µM ethanolamine. At the indicated times the major aqueous catabolites of [^{3}H]serine were isolated from the medium and cells as described in the Material and Methods. Results are expressed as the mean of 3 separate pulse-chase experiments ± S.D.

Compound[a]	Time (min) 0	15	30	60	90	120	240
				(dpm/dishes) x 10^{-4}			
CELL							
E + E-P	2.09±0.76	0.76±0.37	0.60±0.32	0.56±0.41	0.54±0.30	0.54±0.22	0.42±0.23
Serine-P	4.85±2.11	1.76±0.82	1.45±0.84	1.08±0.59	0.85±0.40	0.79±0.44	0.59±0.23
Serine	116.80±45.34	12.53±2.81	8.16±1.77	7.65±1.83	6.14±1.88	5.35±1.73	5.62±0.63
GPE + X	15.68±10.43	12.25±6.51	12.54±7.09	10.61±6.61	9.87±5.72	9.78±5.36	8.19±4.26
GPS	6.63±5.66	3.19±2.57	2.82±2.27	2.19±1.43	2.38±1.76	2.07±1.29	1.78±1.21
Total	146.1±62.4	30.51±9.77	22.57±11.94	22.08±10.79	19.79±7.54	18.55±9.0	16.60±6.40
MEDIUM							
E + E-P	0.61	1.38±0.75	1.65±0.94	2.61±2.37	1.56±0.32	1.86±0.84	2.56±1.39
Serine-P	1.98	3.06±1.22	3.64±1.10	5.05±3.02	3.77±0.90	3.60±2.05	4.93±2.53
Serine	86.05	126.2±40.9	138.0±33.4	128.1±46.0	126.5±34.4	119.3±35.3	142.7±35.6
GPE + X	4.03	6.82±3.52	6.67±3.21	7.86±5.72	6.02±1.41	6.89±2.83	7.36±3.56
GPS	3.13	5.78±3.26	8.16±5.07	11.48±9.30	9.43±4.26	12.35±7.93	16.26±8.82
Total	95.79	143.3±47.9	158.1±42.3	155.1±60.1	147.3±37.9	144.0±48.9	173.8±51.5

TABLE VI.2.

Distribution of [^{3}H]serine-derived label in cellular PS, PE, and PC

Hepatocytes were prepared as described in the Materials and Methods. At the start of the experiment medium was replaced with fresh MEM (serine-deficient) and incubated for 60 min. The medium was then replaced with 2 ml serine-deficient MEM containing 37.5 µCi/ml [3-^{3}H]serine and incubated for 10 min. At the end of the pulse the medium was replaced with MEM containing 300 µM serine.and 100 µM ethanolamine. At the indicated times PS, PE, and PC were isolated from the cells and the distribution of the [^{3}H]-label in the phospholipids was determined after phospholipase C treatment as described in the Materials and Methods. Results are expressed as the mean of 5 separate experiments ± S. E. M.

Percent [^{3}H]Serine-label in Head Group of Phospholipid

Chase Time (min)	PC	PE	PS
0	15.0±10.2	48.5±7.8	91.1±2.6
15	14.4±10.2	39.1±9.5	90.8±1.3
30	13.3±9.7	39.3±10.6	89.9±1.1
60	12.3±8.5	43.3±9.8	90.5±0.8
90	13.0±8.1	47.3±8.9	89.4±0.9
120	12.0±5.9	48.8±8.9	88.1±1.2
240	12.7±5.9	55.6±6.5	87.2±0.6

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CHAPTER VII
DISCUSSION

This thesis was initiated with the goal of obtaining detailed biochemical information about the metabolism of individual molecular species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in cultured rat hepatocytes. When I arrived in Edmonton in August 1987 there were several major breakthroughs which had occurred in Dennis Vance's laboratory that greatly influenced this work. Firstly, it recently had been demonstrated that phospholipids were not homogeneously distributed throughout the hepatocyte, but rather existed in metabolicly distinct pools (Vance and Vance, 1986a). Secondly, Neale Ridgway had just recently succeeded in the purification of the enzyme PE N-methyltransferase (Ridgway and Vance, 1987) and had demonstrated, using HPLC analysis, that this enzyme had little *in vivo* substrate specificity (Ridgway and Vance, 1988). In this environment it became apparent that we could examine the metabolism of the different pools of newly-made phospholipids at the molecular species level. From these studies we have gained new perspectives on the biosynthesis, remodeling, and degradation of PC and PE molecular species, derived from the different metabolic pools.

Phosphatidylcholine

The composition of newly-made PC, derived from either ethanolamine or choline in normal hepatocytes, differed markedly from that of bulk PC (Chapter II). We found that the hexaenoic-rich PC fraction accounted for nearly 25% of the newly synthesized PC, via the CDP-choline pathway, and nearly 60% of the newly-made PC, via the methylation pathway. Over the course of the chase period the newly synthesized PC was actively remodeled to resemble 'bulk PC'. Using the choline-deficient rat model, we were able to demonstrate that the remodeling of PC was regulated in *in vivo* (Chapter V.). The nature of this regulation is unclear but appears to involve both the rates of deacylation and reacylation reactions. The inhibition of PE N-methyltransferase (PEMT) had no effect on PC remodeling and further demonstrated that newly-made PC, as well as 'older' bulk PC, are substrates for the remodeling reactions (Chapter III.). While it had long been established that PC was remodeled

(Lands, 1965) and that remodeling can occur at both the *sn*-1 and *sn*-2 position (Holub, 1980; Thompson and Belina, 1986) the remodeling of the *sn*-1 position has been neglected (MacDonald and Sprecher, 1991). From our studies it appears that the remodeling of hepatic PC, derived from ethanolamine or choline, is a regulated process which involves both the *sn*-1 and *sn*-2 fatty acids. In contrast to these studies, we found that the composition of newly-made PC, derived from serine in normal hepatocytes, does not differ markedly from that of bulk PC (Chapter VI.). Furthermore, we have found little evidence for the remodeling of PC, derived from serine (Chapter VI.). While the lack of remodeling of serine derived PC is surprising, this result is consistent with the observation that the PC pool, derived from serine, is metabolically distinct from PC, derived from ethanolamine and choline (Vance and Vance, 1986a). Taken together, these results demonstrate that different routes of biosynthesis and/or metabolic compartmentation can dictate the metabolic fate of PC.

The possibility that the methylation pathway provided a hexaenoic-rich fraction of the PC in the liver (Glen and Austin, 1971) and synaptosomes (Tacconi and Wurtman, 1985) has been previously suggested but remained unproven. Our studies clearly demonstrate that the CDP-choline pathway produced all the major PC molecular species including significant levels of 16:0-22:6-PC and 18:0-22:6-PC (Chapter II.). Furthermore, we have shown that prolonged inhibition of PEMT with DZA did not signficantly alter the mol% distribution of the major PC molecular species (Chapter III.). In particular, the cellular levels of 16:0-22:6-PC and 18:0-22:6-PC were unaffected by DZA-treatment. If PE methylation provides a specific, hexaenoic-rich, fraction of the cell PC then prolonged inhibition of this pathway with 10 μM DZA should result in the depletion of this fraction of PC. This depletion did not occur. Taken together these results suggest that, in hepatocytes, PE methylation is not necessary for maintenance of PC molecular species composition.

Phosphatidylethanolamine

The composition of newly-made PE, derived from ethanolamine in normal hepatocytes, differed markedly from that of bulk PE (Chapter II.). We found that during the first 2 h of the chase period, PC was a major metabolite of labeled PE. Subsequently, there was minimal conversion of PE to PC which suggests that only newly made PE is available as a substrate for methylation to PC. In contrast to ethanolamine and choline labeled PC, there was little evidence for the remodeling of ethanolamine-labeled PE. Radioactivity disappeared from all molecular species of PE without an increase in any of the species of PE. This radioactivity was recovered in water-soluble metabolites, particularily glycerophosphocholine (GPE), in the cells and medium. Even when PE methylation was blocked with DZA we could find little evidence for remodeling of PE (Chapter III.). This finding was extremely surprising as it had long been accepted that PE was actively remodeled in the hepatocyte (Holub and Kuksis, 1978; Åkesson et al., 1976; Åkesson et al., 1970a; Åkesson et al., 1970b; Trewhella and Collins, 1973; de Tomas, and Brenner, 1970; Holub and Kuksis, 1971). In contrast, studies on the metabolism of newly-made PE, derived from serine, indicated that this particular pool of PE appeared to be remodeled (Chapter VI.). It is interesting to note that the endoplasmic reticulum is the major site of PE synthesis by the CDP-ethanolamine pathway while the mitochondria is the sole site of PE synthesis via PS decarboxylation. Thus, it would appear that the site of PE biosynthesis determines the metabolic fate of the newly-made PE. Recently, Schmid et al. (1991) have demonstrated that PE is remodeled in suspension cultures of rat hepatocytes. Our results are consistant with their findings and are the first demonstration that only specific pools of PE are remodeled in the rat hepatocyte.

Another extremely important result was the observation that 16:0-22:6-PE, labled with [^{3}H]ethanolamine, had an initial turnover rate (5.8 nmol/h) which was two or more times that of any of the other major molecular species of PE (Chapter II.). From these studies it was clear that 16:0-22:6-PE was a substrate for both PEMT and a phospholipase. When PEMT was inhibited with DZA, 16:0-22:6-PE was immediately re-routed from the methylation pathway to the degradative pathway (chapter III.). This phenomenon was not seen for any other PE species and suggested the presence of a 16:0-22:6-PE selective phospholipase A. While *in vitro* studies failed to identify this putative phospholipase (Chapter IV.) the possibility that it exists *in vivo* can not be dismissed. A possible candidate for this enzyme may be one of the recently described high molecular weight, cytosolic phospholipase A$_2$ (Loeb and Gross, 1986; Gronich et al., 1990; Wijkander and Sundler, 1989; Leslie et al. 1988; Diez and Mong, 1990; Clark et al., 1990). Our studies have also demonstrated that newly-made PS is rapidly turned over inside the hepatocyte. As both PS and PE, derived from ethanolamine, are synthesized on the endoplasmic reticulum the same phospholipase may be involved in the catabolism of these phospholipids.

What might be the purpose of a 16:0-22:6 selective phospholipase? Tinco et al. (1969) have suggested that part of the PE, containing the 22:6 moiety, is being recycled. As our results indicate that glycerolphosphoethanolamine is the major PE breakdown product, we propose that the 22:6 moiety is selectively being removed from the 16:0-22:6-PE, resynthesized into 16:0-22:6-diacylglycerol and re-utilized by the ethanolaminephosphotransferase reaction. Such a mechanism would be consistent with the observed rapid turnover of the head group of 16:0-22:6-PE and a hypothesized slower turnover of the 22:6 moiety. This proposal also provides a plausible explanation for the conservation of the docosahexanoate during essential fatty acid deficiency in rats (Yamamoto et al., 1965; Lyman et al., 1969). We also noted that the 16:0-22:6-PC species, derived from ethanolamine was rapidly remodeled (Chapters II and III). Taken together these observations have lead us to postulate the existence of a 22:6 cycle which may ultimately be involved in the production of cellular choline. While it has long been recognized that PE methylation is an auxillary pathway for PC production, often overlooked is the contribution which PE methylation makes to choline production (it is the only significant pathway for choline biosynthesis in animals). It is appealing to speculate that the turnover of 16:0-22:6-PE may regulated by the availability of choline. Thus, similar studies to those conducted in Chapter I should be undertaken in the choline deficient hepatocytes. From our current knowledge we would predict that choline deficiency should increase the flux of radioactive ethanolamine through the 16:0-22:6-PE pool. Another experimental approach would be to demonstrate that the flux of radioactive ethanolamine through the 16:0-22:6-PE pool can be decreased by increasing the extracellular/intracellular choline pool size. As 22:6 may have a particularily important role in the production of choline, the use of an experimentally induced essential fatty acid deficiency rat model in similar studies would be very enlightning.

In conclusion, these studies have not only given us new perspectives on the metabolism of individual pools of phospholipids but have also layed the foundation for future studies that may attempt to elucidate the specific functions of newly-made phospholipid molecules. Futhermore, these studies have demonstrated the value of examining the metabolism of phospholipids at the molecular species level and indicate that too much may be missed by not looking past the level of the phospholipid class.

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